THE BEHAVIOR OF CELLS IN TISSUE CULTURES OF FUNDULUS HETEROCLITUS WITH SPECIAL REFERENCE TO THE ECTODERM.[1]

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INTRODUCTION.

Human, mammalian, avian and amphibian tissues have been frequently employed for the study of cells in tissue cultures but comparatively few observers (Osowski 1914, Lewis 1916 and Dobrowolsky 1916) have used fish tissue. These embryos are easily obtained, the culture media are simple to prepare, and large growth takes place at room temperature. They therefore constitute an ideal material in which to study the movement of the epithelial membrane, the structure of the ectoderm cells and their relation to other cells.

TECHNIQUE.

Pieces of *Fundulus* embryos were explanted into diluted sea water in the manner described by M. R. Lewis ('16). As an additional precaution against infection, the eggs were dropped into 95 per cent. alcohol for one second and then transferred to sterile sea water. In preparing the media several dilutions of sea water were employed: *i.e.*, 20 per cent., 30 per cent., 35 per cent., 40 per cent. and 50 per cent. in distilled water. To 80 c.c. of each of these dilutions was added 20 c.c. *Fundulus* bouillon, 0.02 gm. $NaHCO_3$, and 0.5 gm. dextrose. The media were then sterilized.

Growth was obtained in dilutions of sea water ranging from 20 to 50 per cent., although in the latter it was infrequent and not of great extent. While growth was satisfactory in a dilution of 20 per cent., the proportion of good growths was larger with 30 per cent. and still larger with 40 per cent. sea water. More-

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over, cell proliferation from each piece was more abundant. Cultures grew in media containing no dextrose, or with varying amounts of dextrose up to 2 per cent. Media containing 0.5 per cent. gave the best results. The cultures grew equally well with 80 per cent. of Locke's solution in place of sea water. Good results were obtained with 80 per cent. of Locke's solution and 20 per cent. of chicken bouillon substituted for the fish bouillon, although not so many cultures grew. Pure egg albumen and dilutions of this added to the normal medium likewise proved successful.

Several cultures which contained tissue from both chick and *Fundulus* embryos were made in Locke's solution, chicken bouillon and dextrose. These were kept in the incubator at 39° C. and at the end of 48 hours the explants showed equally good outgrowths.

Cultures were usually prepared in the afternoon, and by the following morning growths in the form of membranes had formed, though sometimes this did not occur until the second day, especially when a dilution of 20 per cent. sea water was used. The embryos were 7, 10, 14 and 15 days old (just before hatching) but the age made no apparent difference in the growth from the explant.

General Characteristics of the Cultures.

Examination of the cultures shortly after explantation usually showed a few isolated mesenchyme cells, which soon began to migrate outward on the cover-slip. The ectoderm cells in the region of the cut edges rounded up into more or less spherical bodies. After several hours typical cultures revealed a membranous outgrowth of ectoderm from one or more regions of the explant, and beyond this an area of mesenchyme cells, isolated, or forming a reticulum upon the under surface of the cover-glass (Fig. 1). Nerve fibers, projecting freely into the fluid along the coverglass, were often present in abundance. Pigment cells and yolk-filled cells from the digestive tract (Fig. 7) were also common. No outgrowth of muscle cells was observed.

A renewal of the fluid medium was not attempted in any of the cultures. The oldest healthy cultures were ten days old,

and in one of these the heart was still beating at the time of fixation. In one culture peristalsis was observed in a portion of the intestine which projected out into the medium. Contractions of the trunk musculature, beating of the heart, and movements of the fins were frequently observed in cultures several days old.

Behavior of Ectoderm Cells at Cut Edges.

In normal embryos the ectoderm consists of a single layer of large pavement cells, polygonal in surface view, which may be clearly seen in the regions covering the fins and the trunk musculature. The cells are transparent and almost colorless. The internal structures are only vaguely visible. The cells covering the trunk have delicate concentric markings, suggesting somewhat the markings upon fish scales.

When the embryos are cut the ectoderm cells along the edge round up very markedly into spherical masses, especially in the region of the heart and the yolk sac. Any small group of cells or single cells that have in cutting become separated from the explant remain for some time in this state. Eventually, large vacuoles develop in them and they remain inactive in this condition for several days before disintegration takes place. Similar cells were frequently observed in old cultures along the edge of the ectodermal membrane.

The direction of the cut is a factor in the successful growth of cultures from the trunk region of the body. The cells do not grow out unless the cut is irregular or oblique. If it is transverse the cells close in and form a covering over the injured end, preventing outward migration. Osowski found that injured surfaces of fish embryos were covered by an epithelial membrane within twenty-four hours.

Membranes never grow out from cut fin surfaces; the cells near the cut surface become wrinkled and irregular in contour, and remain in this condition sometimes for several days, before they become rounded up and display the large vacuoles and greenish protoplasm which are characteristic of degenerating ectoderm cells. Sometimes the ectoderm of the fins degenerates

even when not cut, although the other cells of the culture exhibit normal activity.

Ectodermal Membrane.—Fig. 1 shows the extent of a characteristic membrane in a seven-day culture. In the living cultures the ectodermal cells spread out in a very thin, flat and colorless layer on the under side of the cover-glass. The position of the nucleus and of the granules within the cell could rarely be detected. At the edge of the membrane the cells were thicker and darker in appearance, with a greenish tinge and of very irregular form. Projecting beyond the ectoderm, mesenchyme cells could be seen adhering closely to the cover-glass. Other cells, slightly darker and containing vacuoles and granules, were observed migrating above the ectodermal layer. Slides stained with iron hæmatoxylin show that such outgrowths from the explant consist of a practically continuous ectodermal membrane of extremely flat, slightly granular cells, and a more or less imperfect membrane of mesenchyme lying above it, closely adhering to the cover-glass (Fig. 2). The mesenchymal membrane is never so perfect as the ectodermal layer, and there is a gradual transition from a membranous form to more or less isolated cells which may project for a considerable distance beyond the ectoderm (Fig. 3). One group of ectodermal cells was found which was not covered on its upper surface by mesenchyme (Fig. 8). These cells were characterized by very granular nuclei containing one or more nucleoli, and by mitochondria in the form of threads and granules. Frequently the cells showed a rosette-like arrangement around a small intercellular space. Mesenchyme cells were readily distinguishable in the stained slides by their more granular cytoplasm and indefinite cell boundaries. The size of the nuclei was not a criterion, as the nuclei might be either larger or smaller than those of the ectoderm. Mitochondria were much more abundant than in the ectoderm and stained more deeply. Fig. 9 shows a group of mesenchyme cells, unaccompanied by ectoderm, in which the mitochondria appear very clearly. They were also observed in the living cells when stained with janus green. Certain other granules became visible when stained with neutral red. The latter stain also affected granules in the thickened

cells at the edge of the ectoderm, but the presence of such granules in the flat ectodermal cells could not be determined with certainty. These cells appeared less easily penetrable to a number of vital dyes, while the mesenchyme cells were readily colored.

Formation of Ectodermal Membrane.—In a study of fetal skin growing in blood serum, Loeb ('12) observed that the ectoderm cells migrated into the surrounding medium in the form of strands. Holmes ('13) and Uhlenhuth ('14) described a similar condition in the frog. In my observations on fundulus the ectodermal outgrowths were invariably in the form of very thin, one-layered membranes. The ectoderm never migrated in strands. The earliest appearance of the membrane was indicated by an exceedingly thin, flat layer near the explant which was continuous with the rounded cells covering the body region, and bordered along its outer edge by a broad mass of irregular thickened cells. The mesenchyme cells lay in a thin sheet above the ectoderm and projected beyond it. As the rounded cells migrated from the explant their contour changed gradually, and they became flattened with irregular, thickened, central portions which projected downward in the fluid medium. Text-figure 1 is a drawing of an ectodermal cell in the process of flattening out during its migration. The portion of the cytoplasm spread out along the cover-glass formed a clear thin area bounded by a cell wall which was in close contact with the walls of neighboring cells. In this clear region were a few pale concentric markings. The central mass of thickened cytoplasm eventually disappeared as the cell flattened out completely. A few hours later, as a result of the migration and flattening of the cells, the membrane had increased more than twice in extent, and no thickened cells remained except a few scattered ones and a single row of elongated cells around the edge.

Changes at the Edge of the Ectodermal Membrane.—The edge of the ectodermal membrane was seen to undergo slow and continual changes during the active growth of the culture. Usually the cells were thickened and elongated or extremely irregular in form, with numerous blunt knobs projecting downward. Their cytoplasm appeared granular in contrast to the

apparently homogeneous protoplasm of the flat cells. They were observed to flatten out and thus extend the membrane. It seemed as though the cells were under unequal tension for as the membrane grew wrinkles formed in cells that had formerly been flat. The wrinkles could be differentiated from the cell thickenings previously described, for they were obviously folds in the membrane and often involved more than one cell.

Ectodermal pseudopodia, formed from a hyaline outer region of the cells, have been observed by Harrison ('10) and by Holmes ('13) in tissue cultures of the frog. In the ectoderm of fundulus, however, the entire cell is equally hyaline when stretched out flat, and the formation of pseudopodia was never observed.

The ectodermal membrane is not only very extensible, but elastic as well. In staining the cultures with vital dyes the greatest care was necessary to prevent its retraction, which often followed within a few seconds. This is also likely to occur if the slides are jarred. In one culture the ectoderm cells along the edge contracted and thickened, pulling along with them large portions of the mesenchyme membrane. This double membrane then rolled in upon itself and, as it tore loose from the cover-glass, could be seen adhering by short projections from certain of the cells. These in turn loosened and the membrane pulled in farther (Fig. 5). In such cases of mechanical disturbance of the culture the membrane later became reduced by the contraction of the ectoderm to a compact mass of cells which eventually disintegrated. Ruth ('11) describes the contraction of the edges of the growing epithelial cells during the healing of a wound in the skin of a frog in vitro.

Striations in Ectoderm Cells.—The most peculiar characteristic of the ectoderm cells is the presence of numerous delicate striations, more or less concentrically arranged, which form an intricate pattern over all the cell (Figs. 3. and 10). The markings did not appear on all of the cells and only occasionally were they sufficiently clear in the living cultures to be drawn with the camera lucida. When the cells began to spread out and migrate from the cut surface of the embryo, a few markings could be seen in the flat clear portion of the cell (text-fig. 1) and, as this region

increased in extent, more striations became visible. The markings appeared to be on the under surface of the cells, that is, farthest from the mesenchyme. The striæ were very definite, appearing as longer or shorter dark lines over the cell, varying only slightly in width, and never crossing a cell boundary. The wall between adjacent cells was distinctly double, so that each cell had its own complete investment. This was also clearly seen in a few cases where ectoderm cells had become separated from each other. Frequently one or more striæ near the periphery of a cell were situated parallel with the cell wall, while the inner ones were more irregular or arranged concentrically with reference to several points in the cell.

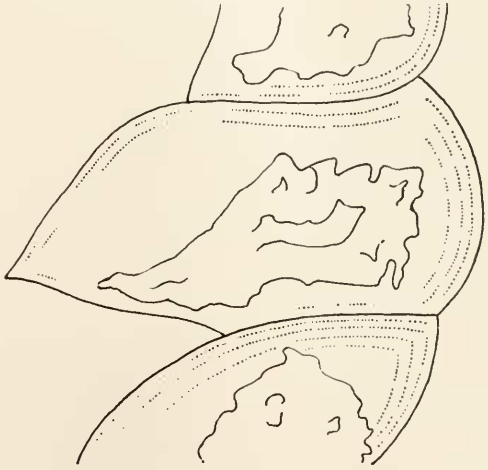

FIG. 1. Camera lucida drawing of an ectodermal cell in process of flattening out during migration; the central mass of thickened cytoplasm bordered by a clear flat region with pale concentric markings. Ocular 5, lens 4 mm.

Form and Behavior of Mesenchyme Cells.—The mesenchyme cells grew out from the explant as more or less elongated and separate cells, which later became connected in various ways. Adjacent cells sometimes sent out short broad processes along their sides, which appeared to fuse with each other, forming a reticulum with relatively small intercellular spaces (Fig. 9). Characteristic also of these cells was the projection of their protoplasm into short thread-like processes often giving a prickly appearance to the edge. The prickly processes became converted

into protoplasmic bridges connecting the cells. When these were present the tissue appeared somewhat similar to the mesothelial membrane described by Lewis and Lewis ('12) in tissue cultures of the chick, except that in fundulus the cell boundaries are much less distinct. Whether the cells actually fuse or not is a question which is extremely difficult to determine with certainty. When the cells are connected with each other by extremely elongated protoplasmic processes, as shown in Fig. 3, the intercellular spaces are large and a wide-meshed reticulum results. There is a gradual transition from a membranous to a reticular arrangement of cells as the distance from the explant increases.

The outward growth of these reticular cells appears to be an important factor in the extension of the ectoderm. As previously noted, reticular mesenchyme cells were usually seen projecting beyond the ectodermal membrane (Figs. 1 and 3). These cells underwent very marked changes in form, their protoplasm flowing in a direction away from the ectoderm, the proximal portions of the cells, however, being connected directly or by intervening cells with the thickened edge of the ectoderm. The distal portions of the cells frequently developed broad fan-like expansions, which were firmly anchored to the cover glass.

The mode of formation of the fan-like expansions appears to be about as follows: The pseudopodia flow out from the cell in the form of delicate, radiating, thread-like or finger-like processes; then the region between the processes gradually fills out until a fan-like form is attained. This soon becomes entirely homogeneous near the periphery and adheres closely to the cover glass. Frequently the fan has a slightly fluted appearance as if not adhering equally well at all points.

As the mesenchyme cells migrate outward they exert a pull upon the edge of the ectoderm which is drawn outward, not by amœboid processes of its own, but by amœboid processes and fan-like expansions of the mesenchyme cells to which it is attached. The sheet of ectoderm is thus anchored in all directions as if by minute guy-ropes. When these are pulled more in one direction, folds and wrinkles are formed, which become smoothed out when the pull is equalized.

Pigment Cells.—Chromatophores of fundulus are large cells containing either black, brownish red, or yellow pigment granules. The cells migrate readily from the explant, but do not as a rule travel far. Stockard ('15) showed that the chromatophores have an affinity for plasma-filled spaces, being found adhering to the pericardium in normal embryos and to the heart itself in embryos deprived of a circulation. In the cultures of fundulus brown chromatophores were several times observed closely adhering to the beating heart, which happened to be projecting out into the fluid medium. Here they remained elongated for several days until the cultures degenerated. Isolated pigment cells that had wandered out upon the membrane degenerated more rapidly than other kinds of cells, and the pigment granules, freed from the cells, were readily taken up by the mesenchymal cells where they became aggregated around the nucleus (Fig. 6).

In addition to the pigment cells and those forming a membrane or reticulum other types of mesenchyme cells were observed. Among these were certain denser, more granular and vacuolated cells which migrated out upon the mesenchymal reticulum, and other cells (probably clasmatocytes) with brighter, more solid-looking protoplasm and numerous curved, finger-like pseudopodia. These cells contained numerous granules often derived from degenerated chromatophores. Brownian movement of these ingested granules was frequently observed.

Yolk Cells.—In some explants certain peculiar spherical cells filled with numerous clear greenish yolk spheres were seen massed together in the anterior part of the digestive cavity. These cells migrated readily out upon the mesenchymal reticulum where they became very slowly amœboid and wandered out along the edge of the ectoderm. Fig. 7 shows a group of these cells with the yolk spheres stained deep black with iron hæmatoxylin.

Cell Division.—The appearance of new cell boundaries in the ectoderm was frequently observed but in no case was a cell seen to divide. There is therefore no evidence as to whether the cells divide by mitosis or amitosis. In over fifty cultures stained with iron hæmatoxylin no stages of mitosis were observed. Two

nuclei, however, were common in ectoderm cells (Fig. 8), as were also nuclei partly constricted into two, or with several irregular constrictions and variable nucleoli. There is no reason to believe that this condition is followed by division of the cells. It may be that the nuclei fuse together again, as Macklin ('16) observed in tissue cultures of the chick, and that the cells subsequently divide by mitosis. Holmes ('13) observed amitosis of the nuclei in tissue cultures of various tadpoles, but he states that nuclear division was not followed by division of the cytoplasm.

A few mesenchyme cells were observed to divide by mitosis and several groups of chromosomes in metaphase appeared in the stained material. Bi-lobed and double nuclei, indicative of amitosis, were also observed (Fig. 9).

Cultures of Chick and Fundulus.—A piece of fundulus tissue and a piece of muscle tissue from a chick embryo of eight days' incubation were placed together in a drop of Locke's solution containing chicken bouillon, and kept at 39° C. At the end of 48 hours each piece showed its characteristic form of growth, the fundulus having the double membrane previously described, the chick tissue showing the usual radiating type of outgrowth. In one region the fundulus outgrowth could be seen growing over a portion of the chick explant as over a foreign body. In another region outgrowths from the two pieces were almost in contact, but the cells from the two explants showed no tendency to intermingle. Specific differences were observable within the cells. Mitochondria are much more abundant in the chick tissues and the cytoplasm appears to be different, as shown by the greater ease with which the cellular structures of the chick may be observed.

General Considerations on the Movements of Membrane Cells.—The behavior of the cells in the cultures at different times is of considerable interest. Taking the normal form of the ectoderm cells as a standard, we find that the cohesive property of these cells is increased suddenly at the time the cut is made, as shown by the rounding up of the cells. The stimulus of the injury was sometimes effective for several hours. This influence seemed to

be gradually weakened and the adhesive power progressively increased as the cells spread out in a thin layer upon the under surface of the mesenchyme. Holmes ('13) found that ectoderm cells of tadpoles in tissue cultures attach themselves readily to various kinds of substrata, including the cover glass, and "extend upon one another in mutual attraction which tends to keep them in continuous masses." In fundulus, however, this stereotropic activity of the ectoderm is called forth only when the cells are associated with the mesenchyme, and it appears to be much stronger than in the frog, causing the cells to be spread out in a thin single-layered membrane. In over fifty cultures it was never observed that an ectodermal membrane grew out unaccompanied by mesenchyme, whereas numerous cultures contained growths consisting of mesenchyme alone. The latter cells appear to be more highly stereotropic than the ectoderm, for they will adhere to the smooth surface of the cover glass even to the extent of having their processes snapped off when the ectoderm retracts.

The difference in behavior of the two layers of cells is perhaps correlated with the fact that under normal conditions of development ectoderm cells grow only in contact with the mesenchyme, whereas mesenchyme cells can grow in contact with widely varying kinds of surfaces.

The question arises whether there is any relation between wound-healing and the formation of the ectodermal membrane in tissue cultures. Loeb ('20) has discussed various processes involved in cell movements in wound-healing, designating among others amœboid migration of ectoderm cells, this being "the first response of the tissue to the wound stimulus." A factor in this amœboid wandering of the ectoderm cells is their stereotropic reaction, as expressed by their contact with the coagulum, which is "the foundation for the process of wound healing." In fundulus cultures the wandering of the cells in contact not with a coagulum but in this case with the mesenchyme layer is the foundation for the process of formation of the ectodermal membrane. The manner of cell movement, however, does not appear to be amœboid in character.

Oppel ('13) has described the bending of the skin edges along the cut surface of explanted pieces of the tadpole's tail as due to real movement; not simply a mechanical process but a change in form by which the ectoderm grows around the cut. In an earlier paper ('12*a*) he compares the ectoderm cells to partly filled sacs of inelastic material which can change their outline without varying the extent of their surface. This kind of movement is strikingly similar to the early changes which take place when the rounded cells begin to flatten. Oppel ('12*b*) distinguishes between epithelial movement, which is a mass movement, and amœboid motion which tends to isolate cells, as in connective tissue. He concludes that while the movement of the epithelium depends on the activities of the cells themselves, it is not an amœboid motion. The observations on fundulus confirm this interpretation. Here epithelial movement appears to be a mass movement throughout all stages in the formation of the ectodermal membrane. Single functional epithelial cells are never found.

In conclusion it may be stated that the activities of the cells in the formation of the ectodermal membrane in fundulus are similar to activities displayed also by ectoderm cells in the process of wound-healing. As in the latter, the cells exhibit mass movements the end result of which is to cover the connective tissue; so in tissue cultures of fundulus the migration of the ectoderm cells proceeds by mass movement which results in a partial covering of the mesenchyme layer. The contact reaction toward underlying connective tissue, exhibited by ectoderm cells in wound-healing, is paralleled by the stereotropic activity of the ectoderm cells evoked by contact with the mesenchyme. It may be said that the cells are attempting to follow out their normal activities, although subjected to abnormal conditions.

Summary.

1. Tissues of *Fundulus heteroclitus* grew in fluid media under conditions varying widely in respect to temperature, concentration of salts, and character of nutritive substances.

2. Mesenchyme cells migrated out into the medium upon the under surface of the cover-glass and formed almost continuous

or reticular membranes with isolated cells lying beyond. The ectoderm formed a membrane in close contact with the under surface of the mesenchyme. Nerve fibers, pigment cells, and yolk cells from the digestive tract readily migrated out. Peristalsis of the intestine, beating of the heart, contraction of the trunk musculature, and movements of the fins, were observed in numerous cultures several days old.

3. Characteristic of the ectoderm cells were certain delicate striations somewhat concentrically arranged, which formed an intricate pattern over the cell.

4. The mesenchyme cells are highly amœboid and possess characteristic fan-like expansions by means of which they adhere to the cover-glass and to each other.

5. While the initial stages in the formation of the ectodermal membrane were accomplished by migration and flattening out upon the under surface of the mesenchyme of the cells originally covering the body, the further extension of the membrane involved the formation and growth of new cells and tension exerted by the mesenchyme upon the thick edge of the ectoderm.

6. Mitosis was observed in several mesenchyme cells but not in the ectoderm, although new cell boundaries appeared from time to time. Frequently ectoderm cells contained two nuclei or one irregularly lobed nucleus.

7. During all stages in the formation of the ectodermal membrane the movement of the cells is a mass movement. Their reactions are much slower than those of the mesenchyme, and are never amœboid in character.

8. There is an essential similarity in the outgrowth of the ectodermal membrane and the process of wound-healing in respect to (*a*) the mass migration of the ectoderm cells, and (*b*) the stereotropic activity of the cells which is evoked by contact with the mesenchyme.

I wish to acknowledge my indebtedness, for the valuable suggestions and criticism, to Prof. W. H. Lewis and Mrs. Lewis, of the Department of Embryology of the Carnegie Institution of Washington, under whose direction this work was accomplished.

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DESCRIPTION OF PLATES.

Figs. 1–10 are from photographs of cultures fixed in Zenker's solution without acid, and stained in iron hematoxylin. All figures except 1 and 5 were made with the high power. 1 and 5 were photographed with No. 4 Oc. and No. 16 lens.

Plate I.

Fig. 1. Seven-day culture from 15-day embryo, showing extent of double membrane composed of ectoderm and mesenchyme. The latter projects beyond the thickened edge of the ectoderm in the form of a loose reticulum or of isolated cells.

Fig. 2. Portion of outgrowth from an explant showing a continuous ectodermal membrane of large flat cells, and an imperfect membrane of darker granular mesenchyme cells, which lies above it.

Fig. 3. Portion of a reticulum of elongated mesenchyme cells, attached at one end to the thickened edge of the ectoderm, anchored at the other to the cover-glass by broad fan-shaped expansions. In some of the ectoderm cells delicate striations are visible.

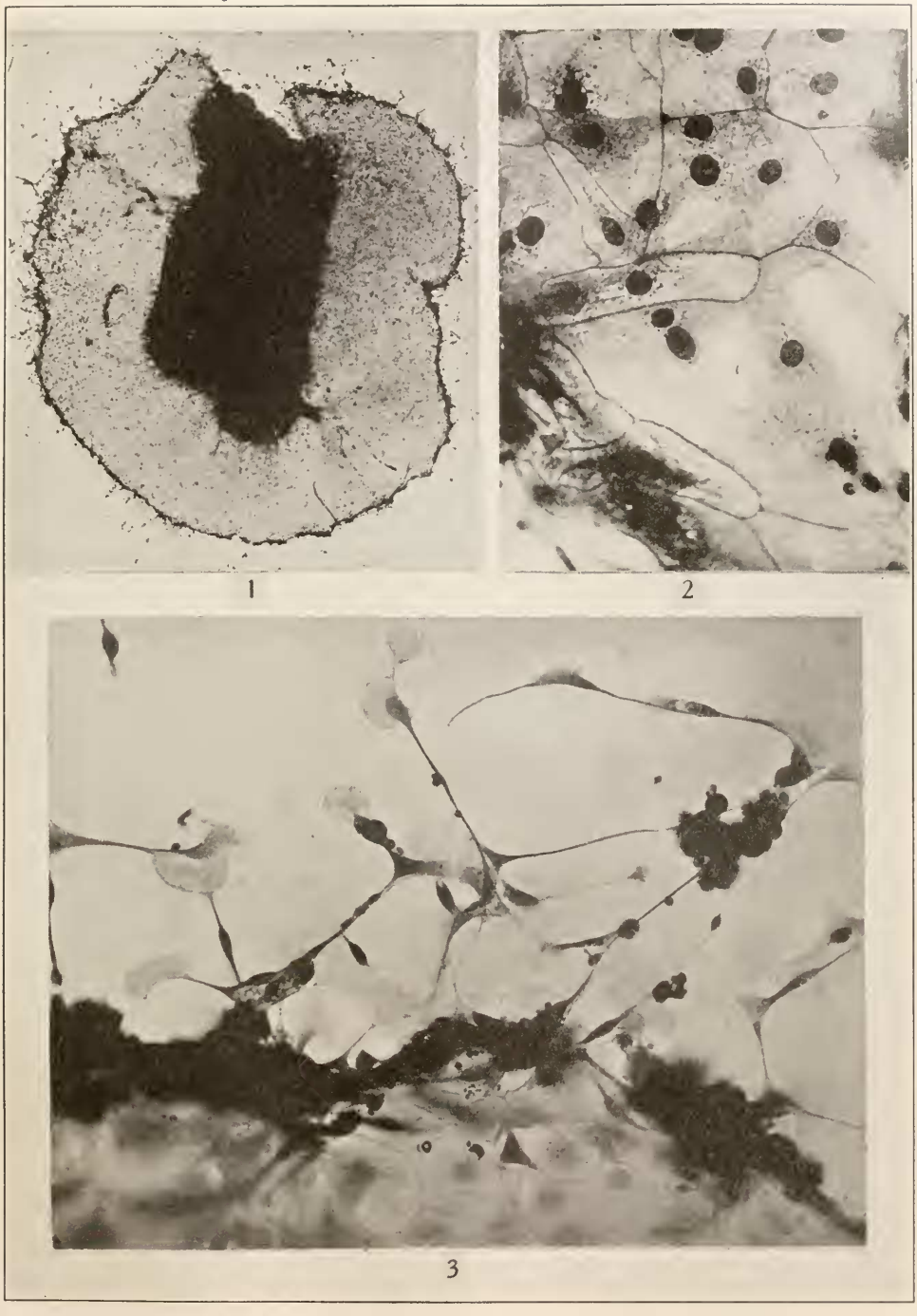

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Plate II.

Fig. 4. Portion of edge of ectodermal membrane, the cells almost completely flattened and slightly curled back at their extreme edge; mesenchyme cells projecting beyond them.

Fig. 5. Result of contraction of the ectoderm due to mechanical disturbance. The dark mass at the left is a retracted membrane of ectoderm and mesenchyme. Other portions of the mesenchyme remained adhering firmly to the cover-glass.

Fig. 6. Group of ectoderm cells, with large nuclei and faintly granular cytoplasm. Nuclei of mesenchyme cells are smaller, and are surrounded by dark granules derived from degenerated pigment cells; the boundaries of mesenchyme cells are not visible.

Fig. 7. Group of yolk cells lying upon the mesenchymal membrane where they had migrated from the digestive tract. The yolk spheres are stained deep black with hematoxylin.

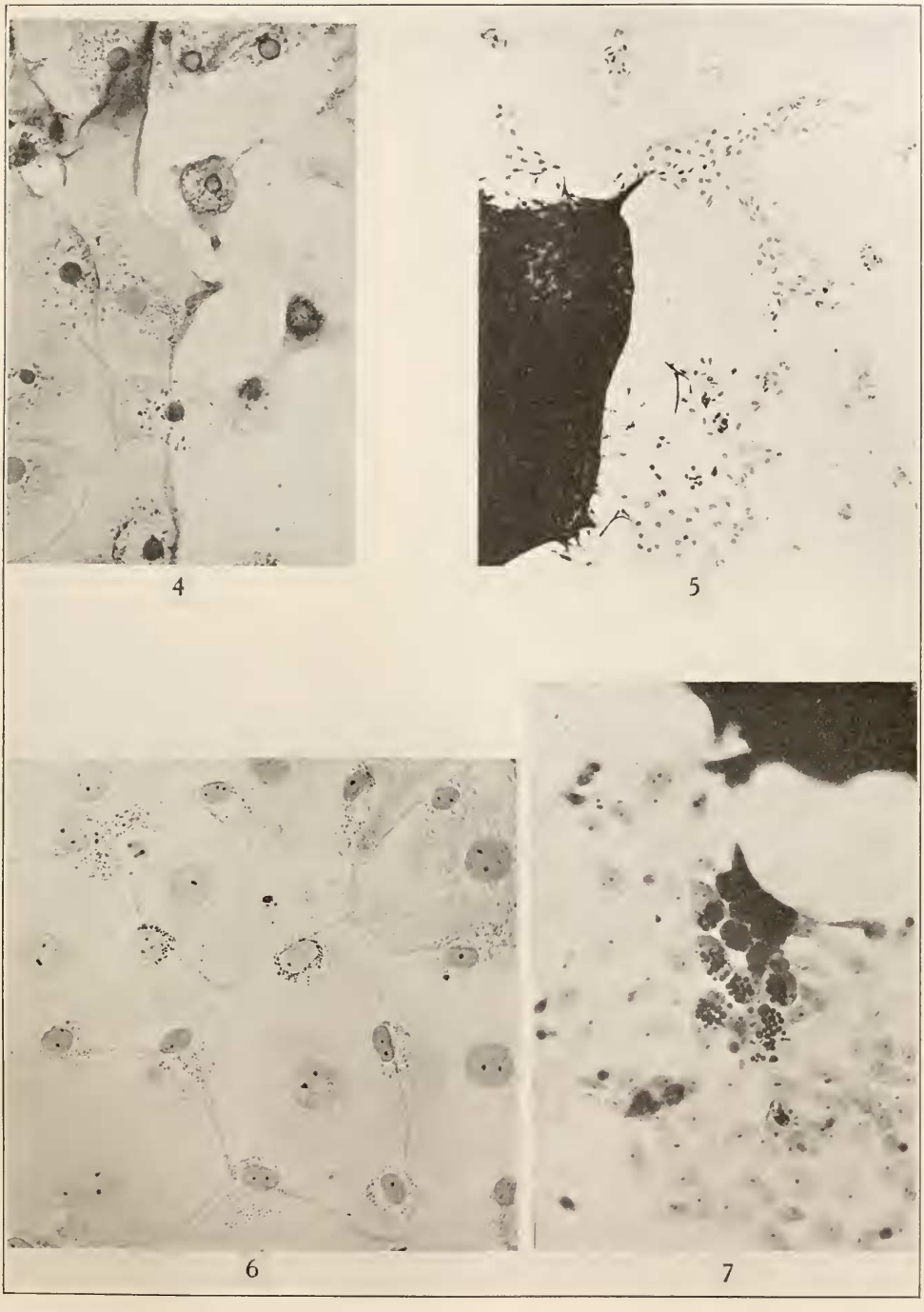

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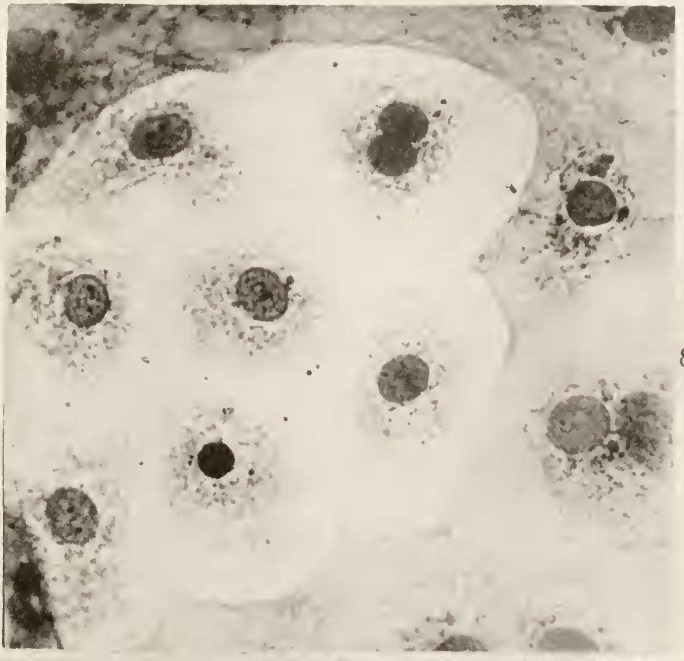
Plate III.

Fig. 8. Group of seven ectoderm cells, free from overlying mesenchyme. A bilobed nucleus is visible in one cell.

Fig. 9. Group of mesenchyme cells forming a membrane with relatively small intercellular spaces. Mitochondria are visible in the form of threads and granules.

Fig. 10. Portion of outgrowth showing striations in ectoderm cells. Nuclei and cytoplasm of mesenchyme cells are visible as dark masses upon the ectoderm.



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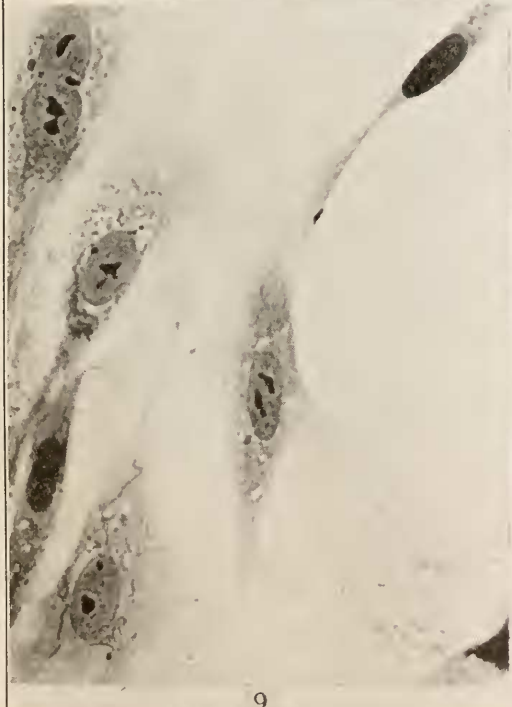

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THE PRESENCE OF GLYCOGEN IN THE CELLS OF EMBRYOS OF FUNDULUS HETEROCLITUS STUDIED IN TISSUE CULTURES.

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At the present time it is practically impossible to demonstrate the various chemical substances of the living cell, owing to the fact that most means of analysis cause the death of the cell. A few of the differential stains, such as Sudan III. and Nile blue, have been applied to studies of living tissues, but these do not behave in the same manner in the living cell as in dead material (Lewis and Lewis, 1915; M. R. Lewis, 1918). In most cases the chemical nature of cytoplasm has been discussed from the standpoint of results obtained from dead cells, and it is doubtful whether such conclusions can be applied directly to the living. This is true in regard to the experiments given herein; for, while the substance described reacts as does glycogen to the tests usually employed to demonstrate glycogen, these results were not obtained until after the cells had begun to be affected by the iodine.

Bernard (1859) demonstrated the presence of glycogen in many kinds of tissues and since that time several methods for showing this substance in the cell have been reported. The methods most frequently used for histological purposes are those of Best (1909), Gage (1917). Neither of these was used in the experiments given below. Instead, the living cells were exposed to iodine vapor in such a manner that they could be followed throughout the experiment, *i.e.*, when living, while dying, and after death had occurred.

Technique.

Small pieces of fundulus embryos (just before hatching) were explanted into hanging drops of the following solution: 80 c.c.

diluted (40%) sea water plus 0.02 per cent. $NaHCO_3$ plus 20 c.c. fundulus bouillon plus 0.5 per cent. dextrose (M. R. Lewis, 1917). Within 24 to 48 hours large growths were present around these explants. The cells composing the growths were then exposed to iodine vapor by scattering a few fragments of an iodine crystal in the bottom of the hollow ground slide under the hanging drop, care being taken to prevent them from touching the drop. The vapor from the iodine crystal penetrated the hanging drop and acted upon the cells. Just a sufficient amount of iodine should be used to rapidly color the cytoplasm yellow and to show the port-wine color of the glycogen within one to two minutes.

Normal Fundulus Cultures.

As has been shown by Dederer (1921), the ectoderm and the mesenchyme cells grow out from explants of fundulus embryos in the form of membranes. The mesenchyme cells are usually attached to the cover-slip and the ectoderm cells form a layer directly beneath them. Mesenchyme cells extend beyond the ectoderm and also along the edge of the membrane and those scattered farther out on the cover-slip are the only cells which have processes to any extent. These processes usually spread out at one end into a large, thin, fan-like structure. One or more large, flat, oval cells, probably endoderm, are sometimes found on the membranes; these are quite different from the mesenchyme cells, in that they have an oval shape while the mesenchyme cells are elongated or somewhat hexagonal, and their cytoplasm also appears to be of a different consistency. For convenience of description these cells will be called *oval* cells. When the cultures were placed over fragments of an iodine crystal the cytoplasm and nucleus of all the cells became yellow, the the mitochondria a darker yellow, and the fat globules a brownish color. Almost the entire cytoplasm of the oval cells, regions of the cytoplasm of some of the mesenchyme cells, and certain parts of a number of their processes turned a port-wine color. This was a distinctly different tint from that exhibited by any other part of the growth and was the same as the characteristic color exhibited by glycogen when exposed to the action of iodine.

The three regions which showed this color reaction will be discussed separately.

1. *Oval Cells.*—As the iodine vapor penetrated the hanging drop, the cytoplasm of these cells became a pale yellow; then the granular portion, including the mitochondria and fat droplets, *i.e.*, the endoplasm, appeared to shrink slightly and became a deeper yellow. Meanwhile the remainder of the cytoplasm, except a thin yellow ectosarc, turned pink, the color gradually deepening until, after a few seconds, this portion was a deep port-wine color, while the endoplasm, nucleus and ectosarc remained yellow. At times the port-wine-colored material occupied the greater part of the cell, leaving only a small clump of endoplasm and the nucleus. In other cases the yellow endoplasm, nucleus and ectoplasm took up practically the entire cell except what appeared as a large port-wine-colored vacuole. The contour of the cell did not change but remained the same size as it was before exposure to the iodine. In a few of these cells there were a number of vacuoles, which also became port-wine color. The color was more intense in tone in the oval cells than in any other portion of the growth. It remained in them for some time, in a few instances for over an hour, then faded, and the whole preparation became a dark yellow color.

2. *Mesenchyme Cells of the Membrane.*—Of the cells forming the membrane probably only the mesenchyme exhibited the port-wine color upon exposure to iodine. This was difficult to determine definitely because the cells adhered together so closely, and also because of the fact that, while the mesenchyme cells were frequently found extended beyond the ectoderm cells, the latter were never observed separated from the former. Not all of the mesenchyme cells showed the port-wine color when in the presence of iodine, and which ones would do so could not be foretold. In these cells the cytoplasm of the central and thicker portion of the cell became a diffuse pink color, while the nucleus and ectoplasm became yellow. This area of pink coloration was not definitely limited but toned off into an extensive yellow ectoplasm. The arrangement of the mitochondria and other structures was the same in the cells having a pink area as in

normal cells. Gradually the pink color became deeper in tone until it attained a pale port-wine color. Within a few seconds the color disappeared from the central region of the cell and a large, round port-wine-colored bleb appeared at one side of the cell. Practically no change was observed in the structure of the cell during the disappearance of the stain and the formation of the bleb. Many of these blebs appeared scattered over the membrane, especially in the region adjoining the explanted piece. The color remained in them for about twenty minutes and then faded out, leaving the blebs rather undefined and difficult to distinguish. In some of the more peripheral cells of the membrane the port-wine color remained diffuse in the cytoplasm of the central portion for some time and then faded out without forming blebs. The mesenchyme cells which had migrated out on the cover-slip, away from the membrane, seldom formed blebs.

3. *Cell Processes.*—The processes of the cells formed the region where the appearance of the port-wine color could be observed most clearly. The large fan-like processes referred to above exhibited lighter and darker areas where the cytoplasm varied either in density or in thickness. After exposure to iodine some of the lighter regions became pink. Later these turned into distinct port-wine-colored areas in the yellow cytoplasm. A few of the processes did not exhibit these stained areas at all; in others some of the areas remained quite pale in color. This phenomenon did not continue for longer than half an hour; at the end of this time the color had faded and that area of the process was slightly shrunken.

When a drop of saliva was placed upon a culture which was later exposed to iodine vapor the port-wine color was not found in the cells. Death of the cell also prevented its appearance. No granules having the characteristic glycogen color were seen in any of the cultures. Neither the mitochondria nor any other granules were concerned in the formation of the port-wine-colored areas. It seemed as though the material which exhibited the typical port-wine color was diffuse throughout certain parts of the cytoplasm and became more definitely localized during the death of the cell, which occurred coincidentally with the iodine

staining. Thus it is seen that some substance, which reacts as does glycogen when exposed to iodine, is present in the cells of fundulus cultures. It is possible that this substance is glycogen. That the substance which became port-wine colored was not the dextrose itself is shown by the fact that dextrose placed in Locke's solution did not so stain when exposed to iodine, while glycogen did.

A few cultures of chick embryos were tested in the same manner for a comparison with those of the fish embryos. No port-wine color was observed in the cells of the older embryos except in one somewhat degenerate culture where a few blebs were already present on certain of the dying cells; these blebs became slightly pink but in no case was there the port-wine coloration such as occurs in fundulus cultures. On the other hand the cells in cultures of very young embryos (48 hours) sometimes contained an abundance of this substance.

The Influence of Starch Upon the Amount of Glycogen Present in the Cells.

Soluble starch (Kaulbaum) was added to the medium of the cultures of fundulus embryos in order to determine whether it could be utilized by the cells to store up glycogen. The starch was dissolved in distilled water and boiled for two minutes; 60 c.c. of the starch solution was then added to 40 c.c. of sea water and the medium prepared in the same manner as for normal cultures. When the quantity of starch was less than 0.1 per cent. it had no appreciable effect upon the cells. In these cultures the growth was normal and no increase in the amount of glycogen could be detected. When larger amounts of starch were added, or when the starch became slightly clumped into masses of very small granules, as sometimes happened, small particles were occasionally taken up by the cell and appeared within the cytoplasm as small granules or granular masses, in some cases surrounded by a vacuole. Upon exposure to iodine the starch became blue, whether within the cell or in the medium. The surrounding vacuole became pale blue, or sometimes lilac, but never the port-wine color indicative of glycogen. The port-

wine color was present in the same regions as in normal cultures, but never greater in amount and sometimes less than in the normal control preparations. Even after a number of days the starch did not become changed into glycogen as, for instance, one 8-day-old culture in 0.75 per cent. starch solution, exposed to iodine, exhibited cells in which there were a few vacuoles some of which contained blue granules. The vacuoles were pale blue or lilac but never port-wine color.

Influence of Dextrose in the Medium Upon the Amount of Glycogen in the Cells.

Cultures were prepared in a medium free from dextrose in order to ascertain whether the lack of dextrose would prevent the appearance of glycogen in the cytoplasm of the cells. The results from twenty such cultures show that, while the amount of glycogen could be decreased by the lack of dextrose in the medium, its presence could not be entirely inhibited. Some of these cultures (48 to 72 hours) exhibited only a slight trace, if any, of the port-wine color when exposed to iodine. On the other hand, a few did contain decided evidences of a small quantity of this substance.

Explants into media containing 0.5 per cent., 1 per cent. and 2 per cent. dextrose, made at the same time as those without dextrose, showed a decided increase in the quantity of glycogen up to a certain point. In no instance did all of the cells of a culture exhibit the port-wine color. Neither did any one cell become greatly filled with this substance. Of all the cultures, those grown in a solution containing 2 per cent. dextrose exhibited the most marked amount of the port-wine-colored material; that is, more cells contained this substance, practically all of the fan-shaped processes had regions which were stained port-wine color, and the color was deeper in tone and did not fade as rapidly as in the normal cultures. Saliva placed upon cultures in 2 per cent. dextrose prevented the appearance of the port-wine color, just as it did in the normal cultures. In these experiments with different amounts of dextrose it was impossible to predict whether a given cell would show the port-wine color in

the presence of iodine. The cytoplasm was not characterized by any structure indicative of this substance, but appeared the same in all of the cells. The processes of the mesenchyme cells had the peculiar lighter areas which in some cases became port-wine colored and in others remained pale yellow.

Summary.

The cells of *Fundulus heteroclitus* grown in tissue cultures contain some substance which behaves in the same manner as does glycogen in the presence of iodine. It is possible that this substance may be glycogen.

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BIOLOGICAL BULLETIN

ON THE INFLUENCE OF TEMPERATURE ON THE EXCRETION OF THE HIBERNATING FROG, RANA VIRESCENS KALM.

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Almost all vital phenomena have been studied as to the influence of temperature on their magnitude. In his excellent book on "Temperatur und Lebensvorgänge," [2] Kanitz gives a summary of the work done in this field, and tries to show that the R-G-T rule of van't Hoff-Cohen holds true also for the reactions of organized material. In Loeb's laboratory it was found by Snyder and Robertson that the heart-beat of invertebrates and of the lower vertebrate follow this rule. These observations have been confirmed and amplified by several authors. For the mammalian heart also some investigators—among them Snyder—tried to show that its frequency follows van't Hoff's rule. Other observers however could not agree on this point and emphasized the complexity of the phenomenon, which would make it improbable that this rule, which holds true for simple chemical reactions, could be applied to these more complex phenomena. The rhythm of breathing, the velocity of conduction in the nerve, the activity of the muscles, the geotropic and phototropic movements of plants, the effect of poisons, the length of life, the rate of development and growth, all have been studied from this point of view, and a great diversity of opinions exists as to whether the rule of van't Hoff holds true or not.

[1] Thanks are due to Mr. R. Goldberg, who made some of the total nitrogen, urea and ammonia determinations for me.

[2] Aristides Kanitz, "Temperatur und Lebensvorgänge," *Berlin. Gebr. Borntraeger*, 1915.

The advocates of the confirming answer (as for instance Snyder and Kanitz) explain the little differences between the theoretical values and the experimental results as due to errors in our methods, while those who deny the parallelism between the reactions of organic material and those of ordinary chemistry emphasize these differences and sometimes claim a regular decrease in the value of the temperature coefficient, as Krogh and Ege did in a controversy with Snyder.

Several authors studied the influence of temperature on metabolic processes. The CO_2 assimilation of plants was shown by Matthaei and numerous other authors to follow the rule of van't Hoff. The frog's metabolism was first studied by Hugo Schulz.[3] The principal importance of his work was that he showed that temperature has a tremendous influence on the frog's metabolism. The frog's output of CO_2 is according to him more than 16 times as much at 25° as at 0°. Aubert[4] gave more accurate figures, but they can still not yet be used for checking them up with the formula of van't Hoff. Vernon's publications,[5] in which he tried to show that between 2° and 17° the CO_2 output was constant, provoked some other papers; the results seem to be due to the sudden changes in temperature to which he subjected the animals. The same fact, a very slow increase in metabolism between 10° and 20°, has also been found in *Cyclodus gigas*, a lizard, by C. J. Martin[6] and in the work of some other investigators.

Experiments.

In my own experiments I tried to determine the influence of temperature on the excretion of winter frogs. Ten animals were kept for 24 hours in a small aquarium. The bottom was covered

[3] Hugo Schulz, "Ueber das Abhängigkeitsverhältnis zwischen Stoffwechsel und Körpertemperatur bei den Amphibien," *Pflüger's Arch.*, 14, 78–91, 1877.

[4] Hermann Aubert, "Ueber den Einflusz der Temperatur auf die Kohlensäure Ausscheidung und Lebensfähigkeit der Frösche in sauerstoffloser Luft," *Pflüger's Arch.*, 26, 293–323, 1881.

[5] H. M. Vernon, "The Relation of the Respiratory Exchange of Cold-blooded Animals to Temperature," *Journ of Physiol.*, 17, 277–292, 1895. H. M. Vernon, "The Relation, etc., Part II," *Journ. of Physiol.*, 21, 442–496, 1897.

[6] C. J. Martin, "Thermal Adjustment and Respiratory Exchange on Monotremes and Marsupials," *Transact. Roy. Soc. London*, (B), 195, 1–37, 1902.

with some distilled water; care was taken to keep this quantity as constant as possible for reasons given in a previous paper.[7] By taking 10 animals at the same time the individual differences were eliminated as much as possible. The aquarium was placed in a larger water container in which the temperature of the water could be automatically regulated, whereas a stirrer moved by a motor kept the water constantly in motion. After 24 hours the urine was centrifuged—to remove the skin particles and the fæces—and then measured. In this urine I ran total nitrogen, urea, ammonia and uric acid determinations.[8] A difference of ten degrees was chosen because the classical formula of van't Hoff's rule speaks of 10°. Higher temperatures than 31° could not be used because a temperature of about 33° is fatal for the frog as appeared in some experiments in which I found all animals dead after having them kept for some time at higher temperatures. Even in the 31° experiments some of the frogs were very faint and near death after 24 hours.[9] This is probably the reason why my figures for this temperature were much more irregular than the rest—in one of the experiments I got for instance 70 mgm. total nitrogen. The results are given in Table I.

TABLE I.

Temperature.	Total Nitrogen.	Urea and Ammonia Nitrogen.	Urea.	Ammonia Nitrogen.	Ammonia.	Uric Acid.	Nitrogen in it.
1°	6.55	6.5	10.5	1.6	1.9	0.0	0.0
11°	10.45	9.2	13.8	2.8	3.4	0.13	0.04
21°	16.3	14.6	23.9	3.5	4.2	0.30	0.10
31°	59.03	50.8	60.4	22.7	27.6	0.83	0.28

[7] H. C. van der Heyde, "Studies in Organic Regulation, I., The Excretion and the Blood-Picture of the Hibernating Frog," *Journ. Biol. Chem.*, XLVI., 1921, p. 421.

[8] The total nitrogen, urea and ammonia figures are the average of three series of determinations on each of which the determinations were made *in duplo*. It should be noted that the figures of one series were not identical with those of the others; but that though the way of increase of each series was identical the absolute values showed some variation. The uric acid figures have only been determined in one series. As previously I wish to state that my trust in the uric acid figures is not very great for reasons given in my previous paper (7).

[9] It seems that the highest temperature which *Rana pipiens* tolerates according to the experiments of Cameron and Brownlee (*Transact. of the Royal Soc. of Canada*, Ser. III., Vol. IX., p. 67) is even lower.

The figures for the total nitrogen are graphically represented in Fig. 1.

It is clear that temperature has in reality a tremendous influence on the frog's catabolism. From 0° till about 20° this increase is only relatively slight. After 20° however the curve rises almost vertically. When we compare our curve with that

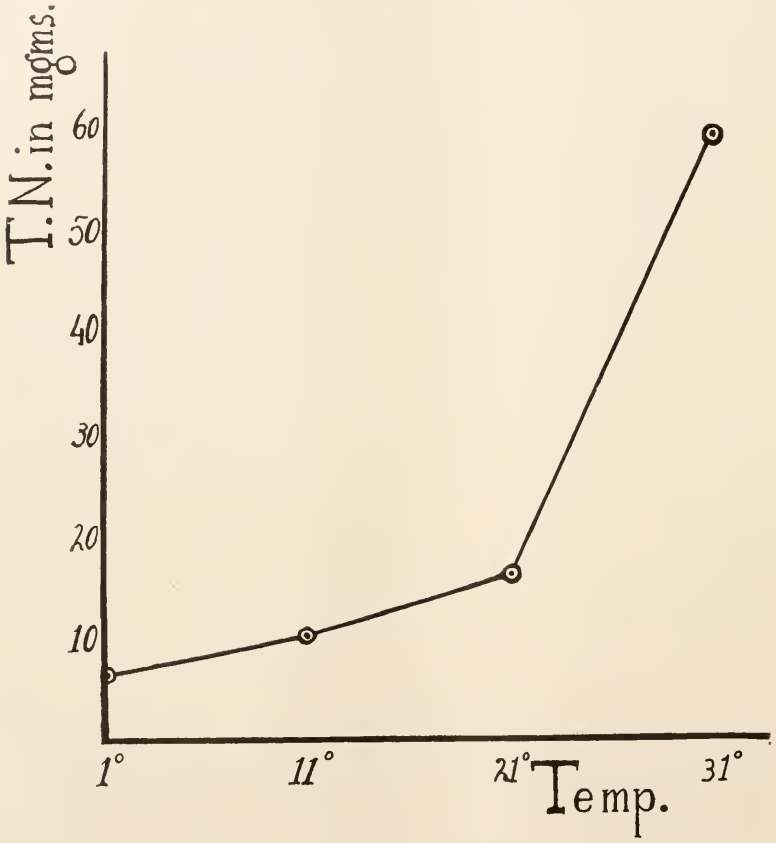

Fig. 1. The influence of temperature on the total nitrogen excretion.

given by Lusk[10] after the figures of H. Schultze, we notice a striking similarity between the two curves. It is clear moreover that this fact has a great biological importance. The frog in his hibernating stage at 4° in the mud can not have the intensive metabolism which the frog in midsummer "as he sits on the

[10] Graham Lusk, "The Elements of the Science of Nutrition," 3d edition, W. B. Saunders Co., Philadelphia and London, p. 115.

river bank and snaps at passing flies" sustains. The change in temperature of its blood causes its tissues to show a much more intensive metabolism. Krogh in his numerous studies on the influence of temperature on poikilothermous animals gave as his opinion that the influence of temperature on the animal's metabolism is of double nature. On the one hand the basal metabolism is increased, on the other hand the tonus of the muscle is increased and causes in that way an increased muscle metabolism. The latter process is regulated by the central nervous system and in fact he observed that in decerebrated animals the temperature did not have as much influence on the CO_2 output as in normal animals. Moreover Krogh is of the opinion that a regular decrease can be observed in the temperature coefficient. For this reason I figured out the temperature coefficients of my own experiments which are given in Table II.

TABLE II.

Range.	Coeff.
1–11°	1.6
11–21°	1.56
21–31°	3.6

Instead of a decrease we notice an increase. We must however keep in mind that these experiments have been made on normal animals. The quoted experiments of H. Schultze give the same result.

I do not dare to say in how far the rule of van't Hoff holds true for this case. I believe that we can not be careful enough in drawing conclusions on this point. The reactions of the organism as a whole can not but with extreme care be compared with simple chemical reactions. Not only the two factors emphasized by Krogh play a rôle in the processes of which we see the final result in our urinary analysis, but also the blood pressure, the water intake which has been shown by Overton[11] to be very strongly influenced by temperature, and the function of the kidneys. For this reason it seems not very probable that the final result of all these processes should be comparable to a

[11] E. Overton, "Neununddreiszig Thesen über die Wasserökonomie und die osmotischen Eigenschaften der Amphibienhaut," *Vorl. Mitt. physik. medizin. Gesellsch.*, Würzburg, N. F., 36, 282, 1904.

simple chemical reaction. When however we calculate the temperature coefficients we see that they are not constant enough to give us the right to the conclusion that van't Hoff's rule holds true in this case, but on the other hand they do not prove the contrary.

To one remarkable phenomenon which I observed in my experiments I might still draw attention. As stated in my

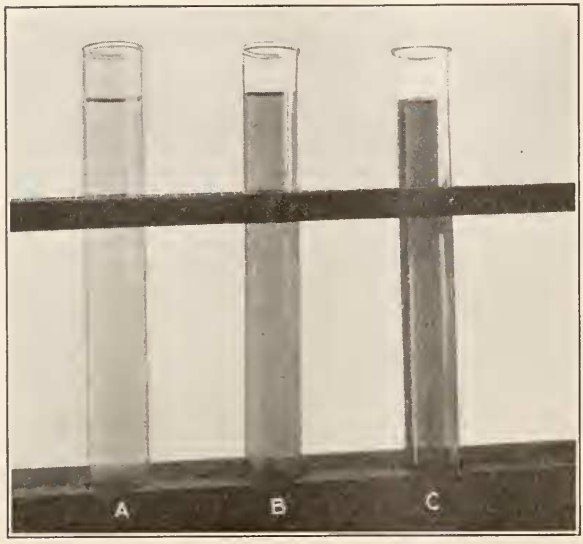

FIG. 2. Influence of temperature on the appearance of the yellow pigment of the frog's urine. *A*, 11°; *B*, 21°; *C*, 31°. As the color of the 1° urine did not differ from that of 11° only these three have been photographed.

previous paper pure frog urine as obtained from frogs in the vivarium in which the average temperature is below 10° is absolutely waterclear. Toda and Taguchi however in their paper on the inorganic constituents of the frog's urine observed a slight yellow color. Their experiments were made on summer frogs. Now it is very remarkable that indeed this pigment appeared in my experiments in which higher temperatures were used. Fig. 2 shows a picture of three samples obtained in three different experiments at 11°, 21° and 31°. The increase in color which was even more striking than this picture shows runs completely parallel with the nitrogen content as represented in Table I. and Fig. 1.

Zusammenfassung.

Die Temperaturabhängigkeit der Stickstoffelimination von überwinternden Frösche (*R. virescens*) wurde studiert. Die Zahlen sind in Fig. 1 graphisch dargestellt. Ein gelbes Pigment tritt auf wenn höhere Temperaturen benutzt werden, die Intensität der Färbung geht dem Stickstoffgehalt und der Temperatur parallel (Fig. 2).

NOTE ON THE PIGMENT OF ARBACIA EGG-SECRETION.

OTTO GLASER.

I.

If the unfertilized eggs of *Arbacia punctulata* are allowed to secrete into a small volume of sea-water, the latter, in the course of five or ten minutes, takes on an amber color. The density of the pigment varies with the concentration of the eggs as well as with the time during which they secrete. After an hour, the sea-water is apt to become reddish-brown.

The later discoloration is due, at least in part, to the elimination of echinochrome—a substance which these eggs contain in considerable quantity. Whether the pigment given off during the earlier moments of secretion is also echinochrome remains uncertain since the diagnostic reactions recommended by MacMunn ('85) are not convincing at the dilutions at which it is necessary to work. In exudate of more than "standard" strength I have been unable to see that HCl produces a red tint or that NaOH intensifies the yellow. With precipitated pigment both acid and alkali seem to intensify the yellow color to an extent barely perceptible.

II.

One cannot assume, offhand, that the pigment is either important or negligible in fertilization. In my study of egg-exudate, therefore, I first attempted to decolorize the secretion in order that the lipolysin and agglutinin subsequently recovered by other methods might be either free or relatively free from pigment. For this purpose charcoal cannot be used since it has the serious disadvantage of removing the whole, practically, of the organic reaction system. Moreover, the pigment, if wanted for separate study, cannot be recovered readily from the charcoal.

To isolate the pigment, it is much better to use chloroform as a decolorant. The method is very simple. To a given volume of

fresh exudate, one adds, roughly, half a volume of chloroform and shakes vigorously for fifteen minutes. In this time an emulsion is formed in which the individual globules are remarkably stable. The system, indeed, is a jelly, white in appearance and surprisingly voluminous. In fact, both its volume and stability at first misled me into thinking that I had found a method for precipitating, if not all, at least the greater part of the organic solutes present.

III.

On standing, the jelly separates from both the unemulsified chloroform and the remainder of the exudate.

The degree to which the latter is decolorized varies, among other things, with its age. If the exudate is perfectly fresh, the pigment is removed almost if not quite completely; if the secretion is 36 or more hours old, decolorization is more difficult and the chloroform jelly less stable.

Microscopic examination of the jelly reveals on the surface of each chloroform globule a delicate skin, translucent, with pearly sheen, continuous, yet also with suggestions of extremely fine fibrils. As the chloroform evaporates, this skin wrinkles until finally there remains an empty bag.

Since the original exudate contained sea-salts, I washed the jelly on a filter or shook it for half an hour in several changes, first of fresh, and later of distilled, water. Under this treatment the globules of chloroform break up into still smaller spheres greatly increasing the stability of the system. The jelly can be freed from sea-salts entirely and in this state has been kept for weeks in stoppered bottles.

IV.

The material in the walls of the globules can be recovered simply by permitting the chlorofrom to evaporate. Slight heat naturally facilitates the process. The jelly, also, may be broken down instantaneously by means of 95 per cent. alcohol. The vesicles are permeable for the alchohol and this, itself, is soluble in chloroform. Since the material held in the walls of the globules is insoluble in both alcohol and chloroform, precipitation is inevitable.

Macroscopically, this precipitate appears to be coarsely flocculent. Its color is yellowish-brown. Under the microscope, granules aside, one sees chiefly fibers. These, when dried on a filter, yield thin felt-like sheets which cannot be readily dissolved in either sea-water or fresh. The material is only slightly soluble in acids and alkalis.

V.

The solubilities of the precipitated pigment are such that it is very difficult to test the importance of this material in fertilization. So far, nothing that would merit particular attention has come to light and the conclusion that properties highly significant in fertilization are absent is reinforced by the eggs of the starfish, the sand-dollar, the oyster, *Nereis* and *Fundulus*, none of which, apparently, secrete anything that corresponds at all closely with the *Arbacia* pigment. However, there is one suggestive fact: after removal of the pigment, the *Arbacia* exudate, physically, is a less stable system than before. From unmodified exudate nothing free from sea-salts can be precipitated with 95 per cent. alcohol; with the pigment removed, 95 per cent. alcohol, insufficient to precipitate the sea-salts, throws down the sperm-agglutinating material. It appears therefore as though the pigment in some way stabilized the exudate.

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Amherst College,
March 1, 1921.

SEX RATIOS IN FŒTAL CATTLE.

F. M. JEWELL.

I. Introduction.

It is a matter of common knowledge that among animals there is an approximate equality of numbers between the sexes, and the possible causes for this equality have long been a matter of speculative interest. Since the dimorphic character of the spermatozoa in various animals including cattle (Wodsedalek '20) has been confirmed by many investigators, it is generally conceded that the sex is determined as soon as the successful spermatozoön enters the ovum.

Therefore, according to the laws of chance, one would expect to find an equality of sexes at the time of conception. However, a vast amount of evidence shows that this is not the case, but that throughout the animal kingdom, with few exceptions, there is a preponderance of males at birth.

In the collection of twins in fœtal cattle used by Dr. F. R. Lillie in his work on the free-martin, the sex ratio was 134 males to 100 females and although the total number was but 108, this represented such a wide departure from the sex ratio at birth, as given by other investigators, as to demand attention. This indicates that there may be either a marked difference in the sex ratios in the fœtal and the born population, or that there is some interference in the chance determination of sexes in dizygotic twinning in cattle.

This investigation was undertaken to determine the fœtal sex ratio in cattle in order that this discrepancy might be cleared up, but more particularly to procure data for comparison of the primary, secondary and tertiary sex ratios as stated by A. M. Schultz ('18).

First it will be necessary to give a statement of the existing data on sex ratios in cattle, expressed as the number of males per 100 females.

Wilckens ('87) gives the ratio in cattle at or near the time of birth as 107.3, the number of individuals being 4,900. Pearl and Parshley ('13) in their studies on the sex ratio in cattle in relation to coitus and the period of œstrus give the ratio as 113.3 in a total population of 480. In more recent data by Pearl ('17) covering 1,313 individuals the sex ratio was 100.12.

It should be pointed out that in other animals the data shows that there is no correlation between the sex ratio and multiple births. Parker and Bullard ('14) and also Wentworth ('14) have shown this to be true for pigs; King and Stotensburg ('15) for rats, and Newcomb ('04) showed that in man the sex ratio in twins was practically the same as in single births.

It should be understood that a different rate of mortality in the sexes either during intrauterine development or after birth would cause the sex ratio to vary at different ages, and for this reason the sex ratio is usually spoken of as primary, secondary and tertiary. The primary is the ratio determined at conception and is the original sex ratio; the secondary is that at time of birth and the tertiary during adult life.

A. M. Schultz ('18) attempted to determine the primary sex ratio for man in an indirect way from the data on the mortality of embryos and fœtuses combined with the sex ratio at birth. In doing this he stated that only in case the mortality of the two sexes was equal would the primary and the secondary sex ratios be equal; that if the male and female abortions were absolutely equal, the sex ratio would be smaller at conception than the secondary, and that if there were a greater intrauerine mortality for males than for females, then the primary sex ratio would be greater than the secondary in proportion to the number of abortions and stillbirths.

In order to determine the primary sex ratio, he ascertained the sex ratio in abortions and stillbirths, and the number of such cases for every 100 living born, both male and female. From the data as given by various writers and from the material that he used (nearly 600 fœtuses of the embryology department of Carnegie Institution) he established the following probable values:

For each 100 living born with sex ratio of.....105.5
8th to 10th month—4 stillborn, sex ratio of.....130.0
4th to 7th month—9 abortions, sex ratio of.....106.3
0 to 3d month—14 abortions, sex ratio of.....125.0
Total conceptions, 127, sex ratio.....“X”

Thus, for every 100 living born he concluded that there were 127 conceptions; 100 with a sex ratio of 105.5, and “a” stillbirths and abortions with a sex ratio “b,” in all with a primary sex ratio “X.” This primary sex ratio he found to be 108.47. Schultz also quotes the determination arrived at by other investigators as follows: Bernoulli, 108.2; Gendrassiks, 108.2; Lenhossek, 111; Auerbach, 116.4 (who believed that it would reach 125 if certain corrections could be made). Schulze thought that it would not exceed 110.

It was more especially in relation to the primary sex ratio and to determine whether there was a different viability in the male and female fœtuses in cattle that the present investigation was undertaken and with these facts as a basis the data obtained are presented. Special acknowledgment is due Dr. F. R. Lillie, who suggested the problem and gave valuable assistance in the interpretation of the data.

II. Data.

A. *Method.*—The work in collecting was done at one of the large packing plants in Chicago during the spring of 1919. In butchering the cattle at the plant, every uterus containing an embryo is taken to a certain room and if the calf is large enough, the skin is saved. Thus the writer was able to open the uteri and record the data directly as each fœtus was removed. In this way any errors or neglect in birth registration are avoided.

The data embraces 1,000 individuals and the sex and length, as an indication of age, were recorded. Observations were also made on the number of corpora lutea in every case where this was possible, especially when twins were found, in which case the position in the uterus also was noted. The complete tabular list with the crown-rump measurement and sex of each individual as removed from the uterus is omitted in this article.

B. *Items of General Importance.*—In regard to the number of corpora lutea, in all of the ovaries examined, about 300 in all,

they corresponded in number to the number of fœtuses with one exception. This exception was a pair of identical twins from one ovum, and since we are concerned with the sex ratio at the time of conception, obviously only one of these should be recorded in the data. In this case both ovaries were present, there was only one corpus luteum, and the twins of course were in one horn of the uterus and both of the same sex. This case of monozygotic twins is of very rare occurrence in cattle, being the first observed in the collection of 108 twins in the Zoölogy Department of the University of Chicago. There were four pairs of twins in the 1,000 fœtuses, and as is commonly the case in twins of opposite sex, the male is usually a little farther along in development than the female. In numbers 646 and 647 the male was 3.5 cm. longer than the female, the latter being 65.0 cm. long. In 807 and 808 the males were both the same length, 68.5 cm. In 988 and 989 the male was 6.1 cm. longer than the female, the latter being 58.2 cm. long.

Twins Numbers 648 and 647 and also 807 and 808 were in separate horns of the uterus and there was one corpus luteum in each ovary; while numbers 988 and 989 were in one horn of the uterus and there were two corpora lutea in the ovary of that side. Since the ovaries on some days were removed for commercial purposes before I had access to them, it was impossible to obtain data of this kind on the total of 1,000 fœtuses.

C. *Analysis of Data.*—Since the length of the embryo can be used as an indication of its age, we can arbitrarily make certain groupings and assume that those within that group are on the average at about the same age.

In Table I. is given such a grouping according to length, from 0–10 cm., 10–20 cm. etc., up to 90–100 cm. The smallest individual was 4.2 cm. in length and the largest was 95.3 cm. in length. Thus the data extends from a comparatively early period in fœtal development practically to the time of birth.

The lengths of embryos were tabulated in lots of 50 in order to get some idea of how the sex ratio would vary according to the position of the group in the total of 1,000 fœtuses. Thus in the first space in Table I. are given the inividuals from no. 1

TABLE I.

THE FŒTUSES GROUPED ACCORDING TO LENGTH. The left-hand column gives the group number in lots of 50. The lowest space gives the totals for the individuals of given lengths.

	0–10		10–20		20–30		30–40		40–50		50–60		60–70		70–80		80–90		90–		
Group.	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	Total.
1.....			1	2	3	2	4	2	1	2	1	3	8	7	4	4	2	4			50
2.....	1		4	1	5	2	5	1	3	3	2	1	1	5	5	7	3	1			50
3.....	2		3	2	2	5		2	1	4	4	3	4	4	6	5	2		1		50
4.....			1		2	2	4	2	4	5	4	1	2	4	7	8	3		1		50
5.....			2	1	5	4	6	1	6	2	2	3	6	3	3	3	2		1		50
6.....	1		1	3	1	2	5	3	2	3	2	3	7	2	6	4	3	2			50
7.....			4	3	5	2		3	4	3	6	4	5	1	6	3	1				50
8.....			5	3	5	3	3	3	5	4	2	4		1	6	2	2	2			50
9.....				4	4	2	7		3	4	2	7	4	3	3	5	1	1			50
10.....	1	2	6	1	3	2	4	5	2	1	6	1	5	2	1	3	2	3			50
11.....	1		4	2	4	5	4	2	4	7	5	4	2	3	1	1			1		50
12.....			2	1	4	6	5	7	7	1	5	1	3	3	3	2					50
13.....			3	2	1	1	4	4	2	2	6	2	7	5	3	5	1	2			50
14.....				2	3	9	5	3	4	5	2	1	4	4	2	1	4			1	50
15.....		1	1	1	5	2	5	3	3	1	4	2	6	3	5	2	3	3			50
16.....			3	3	4	9	6	9	3	2	2	1	3		1		2	2			50
17.....				2	5	3	2	4	6	2	3	4	5	1	2	6	1	4			50
18.....	1	1	7	4	6	6	4	1	3	4	2	2	2	2	1	1	2	1			50
19.....	2		6	3	5	4	6	1	2	3	3	1	1	5	1	1	3	3			50
20.....	2		3	1	5	4	5	5	4	3	3	4	5	2	2	2					50
Total..	11	4	56	41	77	75	84	61	69	61	66	52	80	60	68	65	37	28	4	1	1000

to 50; next, from 50 to 100; 100 to 150 and so on. In the column to the extreme right is given the total for each space as a check on the number of individuals, the total for all spaces being 1,000. The group totals for different lengths, given in the lowest space, show the males without exception to be more numerous than the females, although in the 20–30 group the sexes approach equality. In all, there are 552 males and 448 females, giving a sex ratio of 123.21. If the sex ratio for each of these groups is determined we get the following values (Table II.):

It is obvious that in the 0–10 group and the 90–100 group the number of individuals, 15 and 5 respectively, is too small to be considered separately, especially since the sex ratio in each case is so extremely high. In the 10–20 group the sex ratio of 136.5 is well above the sex ratio of 123.21 for all individuals, while in the 20–30 group it has fallen to 102.6. Then in the next four groups from 30 cm. to 70 cm. the average sex ratio is 127.75.

Therefore, the results in the 20–30 group are very peculiar and can be interpreted only as due to chance, even though the number within that group is 152 individuals. It would be impossible for the males to reach again such a preponderance as 127 to 100 females after such a differential elimination of the sexes as would appear to be indicated in the 20–30 group, if there were such a high mortality of males in that group.

TABLE II.

SHOWING SEX RATIOS ACCORDING TO LENGTH.

Length.	Sex Ratio.	Number of Individuals.
0– 10	275.0	15
10– 20	136.5	97
20– 30	102.6	152
30– 40	137.7	145
40– 50	113.1	130
50– 60	126.9	118
60– 70	133.3	140
70– 80	104.6	133
80– 90	132.1	65
90–100	400.0	5

If the sex ratios are computed for individuals up to a certain length on the one hand and then for all over that length, a comparison can be made of the ratios for *relatively* younger and older fœtuses. In this way if there is any difference in the viability of the sexes we should expect to find that in the younger fœtuses the sex ratio would be high and that there might perhaps be a critical age, as indicated by length, in which there would be a greater mortality for one sex than for the other. We should also expect to find that after this critical stage in development is past the sex ratio in the remaining groups would not vary so greatly from the total of 123 as it did up to that group.

Table III. gives such a grouping with the number of individuals and the sex ratio for each group. The advantage of greater numbers within the group is also gained in this way. The sex ratios in this table were computed from the complete tabular list.

Thus, it is noted that for 112 individuals up to 20 cm. the sex ratio is 148.88 and that for the remaining 888 individuals from

TABLE III.

SHOWING A COMPARISON OF THE SEX RATIOS OF RELATIVELY YOUNGER AND OLDER FŒTUSES.

Length.	Individuals.	Sex Ratio.	Length.	Individuals.	Sex Ratio.
0– 20....	112	148.88	20–100	888	120.34
0– 30.....	264	120.00	30–100	736	124.39
0– 40.....	409	125.96	40–100	591	121.34
0– 50.....	539	123.14	50–100	461	123.78
0- 60.....	657	123.46	60–100	343	122.72
0– 70.....	797	125.14	70–100	203	115.95
0– 80.....	930	121.95	80–100	70	141.37
0– 90.....	995	122.50	90–100	5	400.00
0–100.....	1,000	123.21			

20–100 cm. the sex ratio is 120.34. The objection may be raised, and quite rightly, that 112 is too small a number on which to base definitely the sex ratio for individuals from 0–20 cm., especially since, when the next group of individuals from 20–30 cm. is included, the sex ratio from 0–30 cm. becomes lowered to 120.0. However, it should be pointed out that in the 20–30 cm. group, there is a relatively low sex ratio of 102 when compared with the total of 123, and that this is probably due to chance. When this is taken with the sex ratio of the 0–20 cm. group with a sex ratio of 148, it of course lowers it considerably and the reverse is true for the individuals above that length. Thus, the groups from 30–100 cm., being relieved of the burden of the 20–30 cm. group, have a combined sex ratio of 124.39. This no doubt partly explains such discrepancies as appear in passing from the 0–20 cm. to the 20–30 cm. groups, and in the 20–100 cm. and the 30–100 cm. groups. If the 20–30 cm. group had been such that it could have been considered practically normal, the wide and suddenly marked differences here shown probably would not exist.

However, it might be suggested that, although the sudden drop in the number of males in the 20–30 cm. group may represent a chance occurrence, it might be that this is the critical period in embryonic development in which there is a greater viability in the female fœtuses and that the normal sex ratio for this group might well be below the average of 123.

In the 0–40 cm. group the sex ratio of 125.96 shows a greater

sex ratio than in the 40–100 cm. group with a sex ratio of 121.34, and here again, as in all succeeding cases, the 20–30 cm. group tends to lower the sex ratio for the relatively younger individuals. In the 0–50 cm. and the 50–100 cm. groups there is an approximate equality between the sexes, being 123.14 in the former and 123.78 in the latter case. The sex ratio of the groups, at which so close an approximation is reached, is also practically the same as the sex ratio of 123.21 for the total number of individuals. The same is practically true of the 0–60 and the 60–100 groups with a sex ratio of 123.46 and 122.72 respectively. In the 0–70 cm. group with 797 individuals, there is a slight rise in the sex ratio (125.14) and for the 70–100 cm. group with 203 individuals the ratio is 115.95. In the group from 0–80 cm. with 930 individuals the sex ratio of 121.95 of course approaches the average and in the 80–100 cm. group with only 70 individuals there is a sex ratio of 141.37, which can, in so small a number, likewise be considered as due to chance. From 0–90 cm. with 995 individuals the sex ratio is 122.5 and the remaining five have a sex ratio of 400.

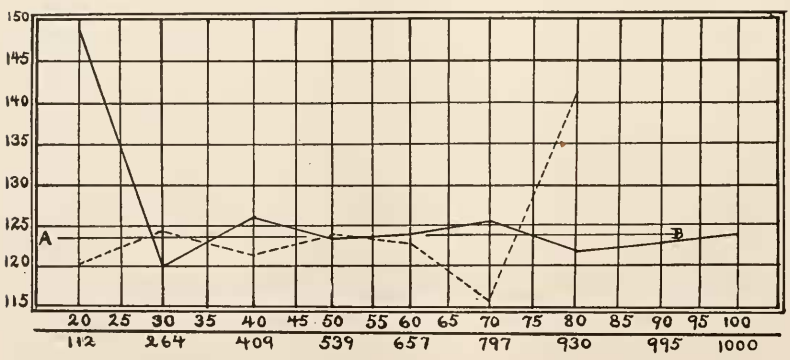

FIG. 1. Representing graphically the sex ratios for relatively younger and older fœtuses. The values for the sex ratios are indicated on the ordinate and the lengths on the abscissa. The number of individuals in each case is given below the lengths. Those below the dividing line (relative to length) are indicated by the continuous line, those below that length by the dotted line.

This is represented graphically in the following figure (Fig. 1) in which the fœtuses below the dividing line (relative to length) are shown by the continuous line.

The values for the sex ratio are indicated on the ordinate and

the lengths of the individuals on the abscissa. The number of individuals dealt with in each case is given below the length. From 30 cm. and upward the highest point is 125.96 in the 0–40 cm. group, and the lowest is 120 in the 0–30 cm. group. The average sex ratio from 30–100 cm. is 123.27 as indicated by the line AB and the average deviation in this group is therefore 2.88.

If the line for all individuals over certain lengths is plotted in conjunction with those below the same lengths, the relative sex ratios for the groups compared can be seen. This is done in Fig. 1, the values being taken from Table III. From this figure it will be seen that on the average the dotted line runs below the heavy line, the former representing the individuals above the lengths indicated and the latter being those below the same lengths. The exceptions to this are in the 20–30 cm. group and the 80–100 cm. group with a sex ratio of 141, which includes only 70 individuals.

Therefore it would seem that in the younger stages there may be a slight difference in the viability of the two sexes, the male being somewhat more susceptible to intrauterine disturbances. However, this difference does not seem to be constant enough to be of real significance and one could not, from the data given here, conclude with certainty that in the fœtal development in cattle there is a greater mortality in the males during any particular developmental stage. A larger collection of individuals within each group would no doubt clarify the situation, although with all groups considered together, the sex ratio of 123 is probably very near to the true value.

Discussion.

A. Reasons for Discrepancies Between the Primary and Secondary Sex Ratios in Cattle

1. *A Possible Differential Viability.*—The only data for the secondary sex ratio with which we can compare the ratios during fœtal development are those of Wilckens ('87) who places the secondary sex ratio at 107.3; Pearl and Parshley ('13) who find it to be 113.3 in a population of 480, and Pearl ('17) who finds it to be 100.12 in a population of 1,313 individuals.

If we can compare the data obtained during fœtal development with that given above as the ratio at birth, it will be noticed that the former on the whole, with a sex ratio of 123.21, is well above the secondary ratio. Since in all of these cases the data include various breeds, it is fair to compare them in this respect. It would thus appear that there is a greater mortality among the male fœtuses, for only in this way would the sex ratio at birth be less than that during fœtal development. The figures as they stand in the present article, however, do not show sufficiently well-marked evidence to support the supposition that there is a greater mortality among the males at any particular stage of development.

2. *Possible Variations in Different Populations or Breeds.*— During the summer of 1919 correspondence was carried on with a large number of breeders to obtain data for comparison of the sex ratio at birth with the ratio during fœtal development, and also to determine the influence, if any, of the breed on the sex ratio. The Short Horn was taken as the beef type and the Holstein-Friesian as the dairy type of cattle. Only registered cattle were used and the data included the sire's name and number, the year, and the number of male and female calves by that sire. The total results of this investigation are given in Table IV. Data were also obtained in reference to twins of the same sex and of opposite sexes.

TABLE IV.

SHOWING THE SEX RATIOS FOR SHORT HORNS AND HOLSTEIN-FRIESIANS.

	Short Horns.			Holstein-Friesians.		
	Males.	Females.	Ratio.	Males.	Females.	Ratio.
Single births	862	881		958	978	
Pairs of twins of same sex	2	2		15	17	
Pairs of twins, opposite sex	8			18		
Single births and twins of opposite sex	870	889	97.86	976	996	97.99
Total, including all twins	874	893	97.87	1,006	1,030	97.66
Grand total, both breeds	Males 1880; females 1923. Sex ratio, 97.76					

From this table it will be seen that the total number of single

births and dizygotic twins in the Short Horns included 870 males and 889 females with a sex ratio of 97.86. The corresponding group in the Holstein-Friesians contains 976 males and 996 females with a sex ratio of 97.99.

In the Short Horns the total including the twins of the same sex, a very few of which *might* have been monozygotic, there are 874 males and 893 females, the ratio being 97.87. The corresponding group of Holstein-Friesians contains 1,006 males and 1,030 females with a sex ratio of 97.66.

The total in both breeds was 1,880 males and 1,923 females, making a total population of 3,803 with a sex ratio of 97.76.

Thus it will be seen that there is no marked variation in the sex ratios of the two breeds, the two in fact approximating each other very closely.

It is possible that in a mixed population there might be a change in the sex ratio as has been indicated by various investigators in data on hybridization. Guyer ('09) found a great excess of males among hybrid pigeons and suggested that "more or less default in the metabolic processes because of the incompatibilities which must necessarily exist between two germplasms so dissimilar" would lead to a production of more males.

M. Pearl and R. Pearl ('08), in comparing pure with cross matings for man in Buénos Ayres, showed a "significantly greater proportionate production of males in the offspring from matings involving different racial stocks than in the offspring from matings in which both parents belong to the same racial stock."

Unusual sex ratios might result in hybridization of breeds of cattle that were unlike in respect to the size of the fœtus. An extreme case illustrative of this would be the bison-cattle crosses. Babcock and Clausen point out that practically all of the offspring of this cross are females due to the increased size of the hybrid fœtus and the consequent abortion or death of the male fœtuses. Thus, sex eliminating factors, as in this case, would change the secondary sex ratio.

Wilckens found that in the "Niederungsrassen" the sex ratio was 114 in 3,009 individuals as compared with an average ratio of 107.3.

3. *Errors in Sampling.*—It is also probable that errors might be made in sampling in the secondary and tertiary sex ratios. This might be done quite unconsciously by farmers, due to failure to record the sex promptly at birth. Some farmers might be prejudiced in favor of one sex and if the calves of the other sex should die before registration they probably would not be entered.

From this viewpoint one might compare the discrepancies in data on the sex ratios in cattle as given by various investigators, ranging from 97.76 (in Table IV. above) to 107.2 in the data of Wilckens and 113.3 as given by Pearl and Parshley.

Numbers Involved.—The question might be raised as to whether the number of fœtuses, 1,000, would represent a small enough number to make a material difference in the sex ratio as compared with a much larger number. Considering the sex ratio in the larger groups of fœtuses when grouped according to length it would appear that the sex ratio for the total collection, 123.21, is very near to the true one during fœtal development in cattle.

B. *Association of Sexes in Fœtal Twins.*

The data indicates that the sex ratio of 134 for dizygotic, fœtal twins, as found by Dr. F. R. Lillie, is not far from the normal of the population and therefore the association of sexes in twins is presumably a random sampling.

Conclusions.

1. The sex ratio during fœtal development in cattle is 123.21

2. This sex ratio of 123.21 during fœtal development compared with the much lower sex ratios at birth indicates a greater mortality of males during intrauterine development.

3. When compared with the sex ratio of 123.21 the sex ratio of 134 in F. R. Lillie's collection of twins in fœtal cattle does not indicate any interference with the chance assortment of sexes in dizygotic twinning in cattle.

4. The data do not indicate that there is any particular stage in development during which there is a more marked mortality among the males than in any other stage.

5. There is no indication that the breed causes any variation in the sex ratio.

6. Discrepancies between the primary and secondary sex ratios in cattle, excepting errors of sampling, are due to sex eliminating factors rather than factors that effect the primary sex ratio.

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A CORRECTION CONCERNING THE LIFE ZONES OF CANADA.

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A great step forward in the study of North American biota was taken when Merriam ('94) divided the continent into life zones and faunal areas on the basis of temperature. His divisions were natural ones and have been widely adopted, though they have been objected to by some botanists who have, however, given us nothing better, or as good. Subsequently several faunal maps of North America have appeared, founded mainly on that of Merriam ('98), and in some of these, notably that of Seton ('09), faunal areas not previously recognized are marked.

All the faunal maps of North America that I have seen are inaccurate in regard to one particular of which I happen to have special knowledge—the border-line between the Transition and Canadian Zones in Ontario. In Merriam's and Seton's maps the top of the Bruce Peninsula is indicated as being in the Canadian Zone, and the line between the Canadian and Transition is a little too low at the point where it touches Georgian Bay. In the American Ornithological Union map (1910) not only the whole of the Bruce Peninsula but a good deal of Central Ontario is indicated as Canadian.

The data which I here make use of in showing the true faunal position of the Bruce Peninsula was obtained while engaged in biological work on the peninsula in 1905, '07, '08, '09, '10, '11, '12 and '15, and that concerning the position of the boundary between the two zones in the vicinity of Georgian Bay was obtained on a motor-boat trip round the bay in 1912, when I camped at various points on the shore, and at Lake of Bays, Muskoka, in 1916. Work done in the vicinity of Ottawa in 1917 confirms the position of the line between the two zones as given by Merriam and Seton.

The Transition Zone, as its name implies, is a region of inter-

gradation between the Canadian and Upper Austral Zones, and is not characterized by a number of species peculiar to itself but by a fauna and flora made up of southern and northern elements. The Canadian Zone, on the other hand, is characterized by many species. It is forested with coniferous trees, in the east mainly with white spruce (*Picea canadensis*), red spruce (*Picea rubra*) and jack pine (*Pinus banksiana*).

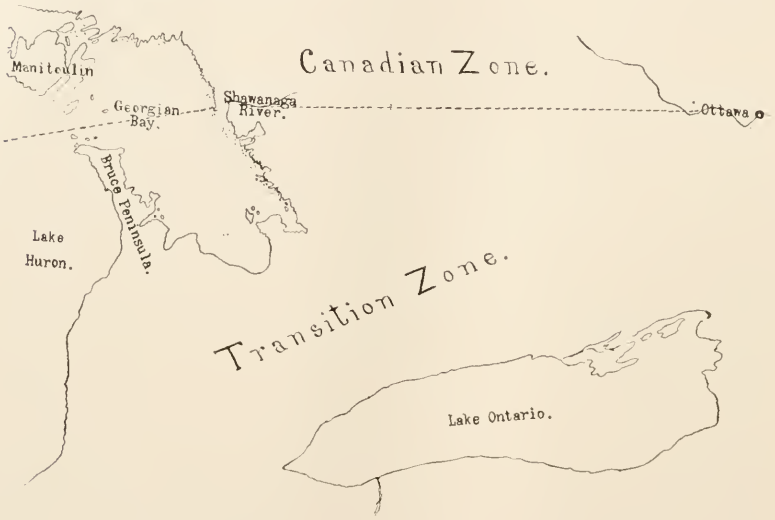
Among the characteristic mammals are the Canada lynx, marten, Canada porcupine, varying hare, northern red squirrel, star-nosed mole, northern flying-squirrel and northern jumping-mouse, and among birds the spruce grouse, arctic three-toed woodpecker, olive-sided flycatcher, Canada jay, American crossbill, white-throated sparrow, slate-colored junco, Blackburnian warbler, Tennessee warbler, magnolia warbler, bay-breasted warbler, myrtle warbler, olive-backed thrush and hermit thrush.

If any region has a flora and fauna consisting of many Austral species and very few Canadian, it is clearly in the Transition Zone. On the Bruce Peninsula, and on Cove, Bear's Rump, and Flower-pot Islands off the top of the peninsula, white spruce occurs but is scarce, red spruce is absent, and the predominating forest is maple-beech. The following Austral plants, which have an extensive range to the southwest, grow on the peninsula—*Gentiana procera*, *Linum medium*, *Satureja glabra*, *Solidago riddellii* and *Cacalia tuberosa*, and also the following species which have an extensive range to the south—*Eleocharis acuminata*, *E. rostellata*, *Scleria verticillata*, *Scirpus lineatus*, *Trillium grandiflorum*, *Viola rostrata*, *Rosa carolina* and *Uvularia perfoliata*. No typical Canadian plants, with the exception of *Carex scirpoidea*, occur on the peninsula, as *Linnæa borealis*, *Cornus canadensis*, *Clintonia borealis*, *Maianthemum canadense* and *Streptopus roseus* which are common, and which are sometimes considered Canadian species, extend far south into the Transition. A fuller discussion of the flora of the peninsula will be found in two of my papers (Klugh, '06, '12).

Among mammals it is the southern wild cat and not the northern Canada lynx that occurs on the Bruce Peninsula, and the Austral form of the red squirrel, *Sciurus hudsonicus loquax*, and not the northern *S. hudsonicus*.

The following Austral birds breed on the peninsula—red-headed woodpecker, Baltimore oriole, towhee, indigo bunting, migrant shrike, brown thrasher, catbird, prairie warbler (see Saunders '06, Klugh '09, '10), wood thrush and bluebird, while of the Canadian birds mentioned above only the white-throated sparrow, Blackburnian warbler, myrtle warbler and hermit thrush breed, the two latter being rare as summer residents.

The above data shows conclusively that the Bruce Peninsula is in the Transition Zone.



It is of course obviously impossible to adequately represent the boundaries between life-zones and faunal areas by a sharp line, since the change from one zone or area to another is gradual and not abrupt. But our lines should be drawn through the region where a comparatively few miles north or south shows an appreciable change in the biota. In the case of the line between the Canadian and Transition Zones in Ontario this is certainly not true if the line is drawn through central Ontario, nor is it true if it touches Georgian Bay low down. It is not until we reach the mouth of the Shawanaga River that we have a clearly Canadian fauna and flora to the north and a Transition biota to the south. That the line should be drawn as indicated on the accompanying map is shown by the data given by Fleming ('01) and Wright ('20) as well as by my data.

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THE PARTHENOGENETIC EFFECT OF ECHINODERM EGG-SECRETIONS ON THE EGGS OF NEREIS LIMBATA.

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Various methods for inducing development in the eggs of *Nereis limbata* have been tried, but, until now, only two have been successful. Fischer ('03), working in Loeb's laboratory, found that *Nereis* eggs would divide and develop to the trochophore stage if treated for half an hour with sea-water which had been made hypertonic by the addition of NaCl, KCl or sugar. Just ('15) obtained the same results by putting the eggs into sea-water warmed to 35° or 36° C. Since all other methods tried had been unsuccessful, *Nereis* acquired the reputation of being refractory to parthenogenetic methods.

Because of this reputation, I decided to see whether or not development could be induced by means of the substance secreted into sea-water by echinoderm eggs. The experiments, as shown in the table, were successful. In performing them, fresh "dry" *Nereis* eggs were allowed to stand ten minutes or more in the secretion and then transferred to sea-water, where a large proportion would exude jelly, form fertilization membranes, and become mature. Some of these, continuing development, would divide, form normal trochophores, and, finally, small worms, in every external appearance like those resulting from sperm fertilization. These have been kept until eighteen days old. It takes these parthenogenetic eggs about twice as long to reach a given stage as it does the sperm controls, a fact which serves as a nice check against accidental contamination with sperm.

Not only is development brought about by egg secretions, but the same effect may be obtained by treating *Nereis* eggs with the lipolysin obtained from the secretions (Woodward, '18).

TABLE I.

	40 sec.		1 min.		2 min.		3 min.		5 min.		7.5 min.		10 min.		20 min.		30 min.		45 min.		50 min.	
	Cleavage.	Swimmers.	Cleavage.	Swimmers.	Cleavage.	Swimmers.	Cleavage.	Swimmers.	Cleavage	Swimmers.	Cleavage.	Swimmers.	Cleavage.	Swimmers.	Cleavage.	Swimmers.	Cleavage.	Swimmers.	Cleavage.	Swimmers.	Cleavage.	Swimmers.
Asterias egg secretion					0	0			0	0			70%	1%	90%	3%	60%	Few	30%	1%		
Asterias lipolysin 1%													30%		40%				81%			
Asterias lipolysin 2%					Few	Few			Many	0			Few	Few	33%	1%			Many			
Arbacia egg secretion			0				20%		10%	1%			30%		30%		10%				1%	
Arbacia lipolysin 1%			1%		5%	1%	10%	Many	8%	Few			1%	.5%	1%	Few	2%	1%			12%	
			0		5%		2%		5%				18%		2%		4%				19%	
Arbacia lipolysin 2%			1%			3%	50%	Few		25%			30%	Few	50%	10%	60%			5%		5%
						Few		Few	Many		Many		Many									
	50%	0	50%	Few	40%	Many	35%	Few	70%	Few	60%	Few	50%	Few								
Echinarachnius egg secretion													50% memb.		10% memb.		10% memb.		16% memb.			
Echinarachnius lipolysin 1%[1]				50%		1%		1%	3%													

The numbers in a horizontal row refer to experiments with a single batch of eggs.

[1] Many of these larvæ were still living eighteen days later.

Since a solution of lipolysin in sea-water may be made much more concentrated than the original secretion, it is not necessary to expose the eggs so long to its action. In fact, one of the most sensational results was the development to the trochophore stage of 50 per cent. of the eggs exposed for one minute to a solution of *Echinarachnius* lipolysin. Most of those eggs continued to develop, and formed segmented worms. The solution used, so-called 1 per cent., was made by dissolving .1 c.c. powdered lipolysin in 10 c.c. filtered sea-water.

In a third series of experiments, dilute *Arbacia* egg-secretion was passed through a Berkfeldt filter. In this process, the agglutinin remains on the filter and the lipolysin, with other substances, passes through. *Nereis* eggs which were left in this filtrate eighteen hours underwent maturation and divided regularly into eight or more cells. They were then transferred to sea-water, in which they continued to develop into normal larvæ.[1]

It was suggested by Dr. F. R. Lillie that the effect of egg-secretion was simply that of a foreign protein, and might be imitated by using cœlomic fluid. Following this suggestion, I subjected *Nereis* eggs to the cœlomic fluid from *Asterias*, treating them with various dilutions and for varying periods of time. While some eggs formed polar bodies and secreted jelly, only a few started to divide, and that very irregularly. I did not succeed in finding any method by means of which the serum would produce normal division or swimming larvæ.

These experiments with *Nereis* eggs continue a series begun several years ago by O. C. Glaser ('14) who found that *Asterias* eggs could be stimulated to divide by letting them stand in *Arbacia* egg-secretion. The following year I performed the reciprocal experiment, which helped to show that the parthenogenetic agent in egg-secretion is not specific. Later, I found that *Asterias* lipolysin produced as many larvæ in *Arbacia* eggs as did *Arbacia* lipolysin (Woodward, '18). The present work shows that the parthenogenetic agent in echinoderm egg-secretions is not even limited in its efficacy to eggs of the same phylum,

[1] This experiment was first performed by Miss M. M. Sampson and repeated by myself.

but that it is effective in bringing about development in some annelid eggs, even though the latter are resistant to the usual agents.

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STUDIES ON BIOLUMINESCENCE.

XIII. Luminescence in the Cœlenterates.

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Introduction.

During a recent trip to the Pacific coast, the opportunity presented itself of studying light production in a number of cœlenterates and of making observations on other luminous forms. These studies were carried out at the Puget Sound Marine Station, Friday Harbor, Washington, and the author expresses his keen appreciation of the facilities afforded him there by Professor T. C. Frye, director of the laboratory.

We are indebted to Panceri (1) for most of our knowledge of luminescence among the cœlenterates. The present paper supplements his work and gives the facts in the case of certain forms found in Puget Sound.

Experiments on Hydromedusæ.

Seven species of hydromedusan jelly-fish were obtained at Friday Harbor, namely, *Æquorea forskalea*, *Mitrocoma cellularia*, *Phialidium gregarium*, *Stomatoca atra*, *Sarsia rosaria*, *Melicerta* sp. ? and an unidentified form. One scyphomedusan, *Cyanœa* sp. ?, is also occasionally to be collected. Of these only the first four produce light and only the first three are markedly luminous. The first five were very common forms, especially *Æquorea*, *Mitrocoma* and *Phialidium*, obtained in the morning from the laboratory float where they live in water of rather low temperature. Their appearance is somewhat capricious, however, and on several days only one or two were seen.

Æquorea and *Mitrocoma*, $1\frac{1}{2}$ to 3 inches in diameter, and *Phialidium*, about 1 in. in diameter, produced light of a bright bluish-green color (but not so blue as the ostracod, *Cypridina*) from spots along the edge of the umbrella at the base of the

tentacles. No other regions of *Æquorea* or *Mitrocoma* phosphoresce, but at times faint light was to be observed coming from masses (gonads) along the four radial canals of *Phialidium*. *Melicerta* is weakly luminous about the manubrium and then only on rather vigorous rubbing between the fingers.

Examined with the microscope in the daytime, the margin of the umbrella of *Æquorea* discloses oval masses of yellow tissue corresponding in position with the luminous areas at night. In *Mitrocoma*, the yellow masses are much closer together forming an almost continuous line in some places. Since the luminous spots observed at night are also very close together in this form, it seems as if the yellow tissue must be luminous tissue. In *Cypridina* and the worm, *Tomopteris*, there are also very clearly visible yellow cells in the luminous gland but this is not true of the luminous organ of all forms. These yellow regions of the medusæ do not stain with neutral red, intravitam, a fact true also for the yellow cells of *Cypridina*. In *Phialidium* or *Stomatoca* yellow cells cannot be made out but this is possibly because of their small size.

Examined at night under the microscope, the luminous spots present a beautiful appearance. Under conditions which cause a cytolysis of the cell, such as addition of fresh water or saponin, one can clearly see that the light comes from granules which are rather large and have a definite boundary,—light discs. They are not mere points of light. They vary in size and will luminesce for some time, then flash out very brightly and the light intensity slowly fade. Sometimes there is the sudden appearance of a light disk and then gradual fading of the luminescence. In the luminous extract of *Cavernularia* (2) I have described a similar phenomenon, where, upon addition of fresh water, the light intensity suddenly increases, due to the flashing out of photogenic granules. Under the microscope the appearance is that of the starry sky.

By addition of saponin to the luminous tissue, we obtain a very bright light and this is the best method of exciting luminescence for examination with the spectroscope. This discloses a band of light extending from about $\lambda = .46\,\mu$ to $\lambda = .60\,\mu$. As far as I

was able to make out the limits are the same for both *Æquorea* and *Mitrocoma*, perhaps a somewhat narrower band for the latter.

The light of these jelly fish only appears on stimulation or on dissolution of the cell. It appears on handling or electrical stimulation, or when the jelly fish is carried by the current against some objects in the water. On merely touching a jelly fish one cannot observe that any luminous secretion is definitely thrown into the water as in the case of *Cypridina*, but on very gentle stroking of the edge of the umbrella a mass of luminous material comes off which adheres to the fingers, or on tossing an animal on the surface of the water, abundant luminous material is liberated which causes the sea water to luminesce. It appears that the luminous material comes off in the slime so commonly secreted by these organisms. A similar behavior is exhibited by the Pennatulid, *Cavernularia*. It would seem that this is to be interpreted as an extracellular luminescence, although not so marked a one, certainly, as that of *Cypridina*. There remains, however, the possibility that we have here cells very easily ruptured, with discharge of their contents into sea water.

The luminous material of *Æquorea*, *Mitrocoma* or *Phialidium* can be dried over $CaCl_2$ and will give a bright light when again moistened.

A strip of the margin of the umbrella of *Æquorea* or *Mitrocoma* is easily cut off with scissors, giving a mass of tissue containing as little non-luminous material as it is possible to obtain. If this is squeezed through four layers of cheesecloth, there is obtained a luminescent extract which glows for some hours. In one case the light was still visible after nine hours. This extract behaves just as a similar one prepared from *Cavernularia* (2). When the luminescence disappears on standing, the addition of fresh water, gentle heating or cytolytic agents such as saponin, sodium glycocholate, chloroform, ether, or NaCl crystals again calls forth the luminescence. Tannic acid, strychnin, or phloridzin do not cause the light to reappear. Once the light has been caused to disappear by addition of saponin or Na glycocholate powder, the further addition of fresh water will cause no more light to appear.

Isotonic cane sugar solution does not call forth the production

of light. The phenomenon is unquestionably one of cytolysis, by diminution of osmotic pressure, by heat, or by addition of specific substances. In the extract there are probably intact photogenic cells which dissolve with production of light. In addition I believe the solution of photogenic granules is also accompanied with the emission of light, because one can very easily see, under the microscope at night, the sudden appearance of a disc of light, too small to be the illumination of a cell, but capable of interpretation as the light from a single granule within the cell.

Extracts of *Æquorea* which should contain luciferase give no light with extracts of *Æquorea* which should contain luciferin. The same is true for *Mitrocoma* and for crosses of luciferin and luciferase of *Cypridina* with these two medusæ. Every attempt to demonstrate these substances has given negative results. The reasons for this are discussed in another paper, to appear shortly.

It is reported that many luminous forms produce no light in the daytime, the power only appearing with the approach of dusk or if the animals are kept in the dark for some time. This is true of some forms but not of these medusæ. The four kinds of luminous medusæ, *Æquorea*, *Mitrocoma*, *Phialidium* and *Stomatoca*, were collected in bright sunlight and brought by an assistant to the dark room where I had been adapting my eyes to the dark for one half hour. All four forms luminesced immediately on stimulation and just as brightly as at night. The ctenophore, *Bolina*, did not luminesce even after ten minutes, when brought into the dark from strong sunlight, but did luminesce after thirty minutes. There is no doubt that *Bolina*, a further discussion of which follows, is affected by sunlight but these four medusæ are certainly not. *Noctiluca* appears to have its luminescence inhibited by strong sunlight also.

Experiments on Ctenophores.

At Friday Harbor, three species of ctenophores occur, *Bolina* sp. ?, *Pleurobrachia* sp. ?, and *Beroë* sp. ?, but only the first was common during my stay. *Bolina* luminesces readily at night. *Pleurobrachia* did not luminesce even on crushing and *Beroë* only gives a diffuse flash of light on vigorous agitation.

The light of *Bolina* comes from cells along the swimming plates. According to Dahlgren (3), the luminous cells form a layer over the testis and ovary, along the water vascular canals. In the living animal I was unable to make out any yellow cells in this region, comparable to the yellow masses of *Æquorea* or *Mitrocoma*.

The light is of the same bluish-green color as the medusæ, but too faint and evanescent for a study of its spectrum.

Bolina is an exceedingly fragile ctenophore and contains much water and relatively little luminous material. The animals also appear to be easily fatigued and lose somewhat their power to luminesce on frequent agitation. Portions of the swimming plate tissue placed on a glass slide, as much of the water drained away as possible, and dried over $CaCl_2$ in the dark do not give light on again moistening with water. This is probably to be explained by the small amount of photogenic material present.

If Bolinas are pressed through four layers of cheesecloth there is obtained a luminescent solution which rather readily loses its power of luminescence. It again gives light on vigorous agitation or addition of cytolytic substances. It behaves as the extracts of medusæ and pennatulids. If fresh water is added, we have the appearance of dots of light just as in these extracts. The existence of luciferin and luciferase also cannot be demonstrated and extracts of *Bolina* give no light with *Cypridina* luciferin nor do heated extracts of *Bolina* give light with *Cypridina* luciferase.

Since the observations of Allman (4) it has been known that ctenophores would not produce light in the daytime. Peters (5) made quite a study of this in *Mnemiopsis* and found that mechanical stimulation accelerates the appearance of luminescence in darkness after previous exposure to light. The inhibition of luminescence is roughly inversely proportional to the intensity of the light which has previously illuminated them.

Bolina shows marked inhibition of luminescence as a result of previous illumination. Animals brought into a dark room from direct sunlight about 10 A.M. gave no light whatever on stimulation immediately or after five minutes, gave some light on stimulation after ten minutes, and a good luminescence after one half hour in the dark. The question at once arises as to the cause of this behavior. Are the cells incapable of being

stimulated after exposure to sunlight or do they fail to manufacture photogenic substances as a result of exposure to sunlight? One alternative supposes the cell to contain photogenic material which for some reason cannot be oxidized; the other, that no photogenic material is formed in the sunlight and the disappearance of that which has been formed. Some evidence can be obtained for the latter view by breaking up the cells of ctenophores which have been previously exposed to daylight. If no luminescence is produced the effect of light must be to prevent the manufacture of photogenic material. If luminescence occurs on breaking up of the photogenic cells previously exposed to daylight, the inhibitive actions of light must be on the stimulation mechanism.

If Bolinas, which have been previously exposed to daylight, are crushed through four layers of cheescloth, no light whatever appears during the crushing or on adding fresh water to cytolyse the photogenic cells. Similar Bolinas, kept in the dark for one half hour, give a bright luminescence under the same treatment. If this extract of crushed Bolinas, which had been previously exposed to sunlight, is allowed to stand in the dark for one half hour and then fresh water added, no light will appear. Whole Bolinas after sun illumination will again luminesce if kept in the dark for one half hour. This shows that there is no preformed photogenic material in sunlight exposed Bolinas and that none can be formed in crushed material even in the dark. The sunlight must therefore act to prevent the formation of photogenic substance rather than to prevent its oxidation on stimulation. Why sunlight causes the disappearance of photogenic material already formed is a question awaiting solution.

Experiments on a Sea Pen, *Ptylosarcus*.

Ptylosarcus is dredged at Friday Harbor in fairly deep water. Some of the specimens may be two feet long. The colony consists of a stalk without fronds buried in the sand and a stem with fronds that bear polyps only along the outer edge. The polyps, but not the surface of the fronds, are luminous. The stalk is not luminous but the stem has two luminous areas running the length of it and one non-luminous area between these.

The colony is non-luminous except when stimulated. Then a yellow greenish light appears of a more yellow hue than *Æquorea*. If the polyps are gently rubbed, a luminous slime comes off and the secretion can be seen in the sea water. The polyps, ground in sea water with sand, give a luminescent secretion which becomes very brightly luminescent on addition of fresh water, saponin and other cytolytic agents. The extract behaves exactly as a similar one prepared from *Cavernularia* and already described (2). In every way the behavior of *Ptylosarcus* agrees with that of *Cavernularia*.

It was impossible to demonstrate the presence of luciferin or luciferase in *Ptylosarcus*. The following "crosses" were also made, using extracts, which, from mode of preparation, should have contained *Ptylosarcus* luciferin and luciferase.

Ptylosarcus luciferase × *Ptylosarcus* luciferin—negative.
Ptylosarcus luciferase × *Æquorea* luciferin—negative.[1]
Æquorea luciferase × *Ptylosarcus* luciferin—negative.
Cypridina luciferase × *Ptylosarcus* luciferin—negative.
Ptylosarcus luciferase × *Cypridina* luciferin—negative.

Ptylosarcus brought into a dark room from direct sunlight was observed to luminesce on immediate stimulation and as brightly as at night. There is no inhibitive influence of light in this form.

Experiments on a Sponge, *Grantia*.

The question of luminosity in sponges is in rather of an unsettled state. Some observers have reported luminescence but Dahlgren (3) was inclined to attribute the light of a sponge obtained at Naples to luminous worms and protozoa living in its canals.

At Friday Harbor there exists a sponge, *Grantia* sp.?, one to three inches long, common on logs, piles, etc., in the sea water. If rubbed, a yellowish luminescence may be observed which can be obtained from all parts of the organism. If the sponge is crushed the luminescence is quite bright. Every individual of this kind of sponge examined showed luminescence, whereas another sponge, *Esperella* sp.?, living on *Pecten* shells, was not luminous. A few isolated dots of light only appeared on rubbing.

[1] Sometimes a faint light was observed whose significance is unknown.

Sponges kept in sunlight for one half hour gave as good a luminescence as those in the dark.

Whether this is a true luminescence or whether due to luminous organisms living on the sponge cannot be definitely stated. The sponge could not be stimulated to luminesce electrically (interrupted induced currents) under conditions when jelly-fish showed a good luminescence. Examined under the microscope, no hydroids, radiolaria, dinoflagellates or Noctilucæ could be observed, but many desmids, diatoms, worms and infusoria. These forms are not luminous, however.

When squeezed through cheesecloth a luminous extract was obtained, the light coming from points of light in the extract as in the case of *Cavernularia* or medusæ. Addition of fresh water or saponin causes a great increase in light just as in extracts of cœlenterates. No luciferin or luciferase could be demonstrated.

The fact that an extract of this sponge made by squeezing through cheesecloth remains luminescent for some time is significant. If the light came from small luminous forms living on the sponge, we should expect them to pass through the meshes of the cheesecloth unharmed and then light would appear in the extract only on stimulation by agitation or in some other way. It is also significant that no inhibitive effect of sunlight was to be observed. Sunlight is said to inhibit the luminescence of many small organisms, especially dinoflagellates, which might live on the sponge. In general characters, the extracts so closely resemble those obtained from cœlenterates that I am inclined to believe the light of this species of sponge is a true luminescence.

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THE EXPLOSION OF THE SPERMATOZOA OF THE CRAB *LOPHOPANOPEUS BELLUS* (STIMPSON) RATHBUN.

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(FORTY-SIX FIGURES.)

CONTENTS.

INTRODUCTION.

For a number of years the writer has been studying the male germ cells of the Decapoda with two purposes in mind: (1) to discover the means by which the mature, dormant spermatozoa of the Decapoda become activated, in order to shed light on the problem of fertilization in this order of Crustacea, and (2) to trace more clearly the process of spermatogenesis. The present paper on the explosion of the spermatozoa of the black-clawed crab, *Lophopanopeus bellus*, is a contribution involving the former of these problems.

MATERIAL AND METHODS.

The material for this study consisted of the living spermatozoa of *Lophopanopeus bellus*, common in certain localities around the Puget Sound Biological Station, Friday Harbor, Wash. The spermatozoa of this crab are very favorable for study in that they are not enclosed by the numerous spermatophores so common in other brachyura. As pointed out in another paper (Fasten, 1917), "in *Lophopanopeus bellus* it doesn't seem as if numerous spermatophores are developed. Here it appears that one large spermatophore is formed in which the spermatozoa are tightly packed." Since this is the condition all that was necessary to

obtain a plentiful supply of living spermatozoa was to rupture the deferent ducts and the male gametes oozed out in tremendous numbers.

The living spermatozoa were studied in the same manner as described in my earlier paper on the spermatogenesis of the edible crab, *Cancer magister* Dana (Fasten, 1918). Numerous spermatozoa suspended in the crab's body fluid, or in sea water which is isotonic with the crab's body fluid, were placed on a slide and covered with a cover glass. These could then be studied with the high power oil-immersion lenses. By allowing various chemical solutions to diffuse under the cover glass all changes in the spermatozoa could be observed and outlined with the aid of the camera lucida.

The living spermatozoa were studied in the following solutions:

1. Crab's body fluid.
2. Sea water.
3. Sodium chloride (NaCl)—M/2 NaCl and less.
4. Sodium nitrate ($NaNO_3$)—M/2 $NaNO_3$ and less.
5. Calcium chloride ($CaCl_2$)—3/8M $CaCl_2$ and less.
6. Potassium chloride (KCl)—M/2 KCl and less.
7. Potassium nitrate (KNO_3)—M/2 KNO_3 and less.
8. Potassium hydroxide (KOH)—very dilute solution.
9. Distilled water.
10. Cane sugar ($C_{12}H_{22}O_{11}$)—M/1 $C_{12}H_{22}O_{11}$.
11. Ovarian fluid.
12. Acidulated sea water. Various small amounts of acids were added to sea water, such as: glacial acetic, salicylic, saponin, sodium glycocholate, nitric, hydrochloric, oxalic, tannic, picric, and chromic acids.

Many of the spermatozoa in all stages of explosion were fixed on the slide with either osmic acid fumes, or Bouin's fluid, or Flemming's mixture, and then stained with Heidenhain's iron-hæmatoxylin and acid-fuchsin. Those fixed with osmic acid fumes gave beautiful results, so that the stained elements were perfect representations of the living structures. This can be clearly seen when one examines Figs. 3–7, which are from stained preparations fixed with osmic acid fumes, and compares them with figures 1 and 2 which are from living spermatozoa suspended in the body fluid of the crab.

Normal Appearance of Spermatozoa.

The living spermatozoa of *Lophopanopeus bellus* when studied in the cœlomic fluids of the crab are found to be small, greenish bodies, which appear like spheroids when seen from the top or bottom (Fig. 1), and like ellipsoids when viewed from the side (Fig. 2). In structure they seem to be similar to those of *Cancer magister*. Within the centre there is a clear central body (Figs. 1 and 2, *b*) and surrounding this are two vesicles; a uniform, darkly green secondary vesicle (Figs. 1 and 2, *v'*), and a clear, transparent primary vesicle (Figs. 1 and 2, *v*). Outside of these vesicles is a granular and vacuolated protoplasmic cup (Figs. 1 and 2, *h*) of a lighter greenish hue than the secondary vesicle. If the spermatozoa remain suspended in the crab's body fluids for some time their protoplasmic cups open up and liberate the radial arms (Figs. 3–7). It is thus seen that the protoplasmic cup of the spermatozoön consists of a nuclear cup (Fig. 3, *n*) and radiating radial arms (Fig. 3, *r*).

When the spermatozoa are fixed with osmic acid fumes and stained by the iron-hæmatoxylin and acid-fuchsin methods, then the nuclear cup, radial arms and the central body stain black (see Figs. 3–7), the second vesicle stains a dark amber, whereas the primary vesicle remains transparent.

Four types of spermatozoa are produced, depending on their number of rays. There is a three (Fig. 4), four (Fig. 5), five (Fig. 6), and a six (Fig. 7) rayed type. The four (Fig. 5) and five (Fig. 6) rayed types, however, are produced in largest numbers. These rays are not pseudopodia-like processes similar to those which Binford ('13) pictures for the spermatozoa of *Menippe mercenaria*. They are distinct arms similar to those found in the crayfish *Cambarus virilis* and *Cambarus immunis*, as pictured by the writer in a previous paper on the spermatogenesis of these forms (Fasten, 1914).

Effects of Chemical Agents on Spermatozoa.

1. *Sea Water.*—Sea water produces no change in the normal appearance of the spermatozoa. The protoplasmic cup, however, swells slightly and liberates the radial arms (Figs. 8 and 9).

2. *Sodium Chloride.*—An M/2 NaCl solution which is isotonic

with sea water produces no change in the normal appearance of the spermatozoa (Figs. 10 and 11). An M/4 NaCl solution brings about a slight shrinkage in the nuclear cup, otherwise there is no further change. In an M/6 NaCl solution the secondary vesicle is very slowly everted. First of all it squeezes out in the form of a small bubble (Fig. 12), until very gradually it assumes the appearance shown in Fig. 13. In an M/7 NaCl solution the eversion of the secondary vesicle is much faster. Figs. 14, 15 and 16 show successive stages in the eversion process. Most of the spermatozoa proceed to the stage shown in Fig. 16 and then cease. An M/8 NaCl solution brings about a complete and rapid explosion of all the spermatozoa. Figs. 17, 18 and 19 show respectively the beginning, middle and end of the process. In Fig. 19 the secondary and primary vesicles, as well as the central body are seen completely everted.

3. *Sodium Nitrate.*—An M/2 $NaNO_3$ solution which is isotonic with sea water brings about no appreciable change in the normal appearance of the spermatozoa. In an M/4 $NaNO_3$ solution the only change noticed in the spermatozoa is a slight swelling of the nuclear cup. An M/8 $NaNO_3$ solution causes a slow eversion of the secondary vesicle, producing figures similar to those shown in Figs. 15 and 16. In an M/16 $NaNO_3$ solution the eversion of the two vesicles occurs rapidly and with considerable force, so that all the spermatozoa soon take on the appearance shown in Fig. 19.

4. *Calcium Chloride.*—A 3/8M $CaCl_2$ solution is isotonic with sea water and this brings about no change in the normal spermatozoa. A 3/11M $CaCl_2$ solution brings forth a partial eversion of the secondary vesicle (Fig. 20). In a 3/16M $CaCl_2$ solution the spermatozoa explode completely. The vesicles are entirely everted and at the same time the nuclear cup shrinks considerably and becomes irregular. Figs. 21–24 show various stages in the explosion process. In the $CaCl_2$ solutions the detailed structure of the spermatozoa can be clearly distinguished.

5. *Potassium Chloride.*—In an M/2 KCl solution which is isotonic with sea water the spermatozoa remain normal. In M/4 and M/8 solutions of KCl the only perceptible change produced in the spermatozoa is a disappearance of the granules

and vacuoles in the nuclear cup making it become more homogeneously green. Also the secondary vesicle shrinks somewhat, thereby leaving the clear primary vesicle to show more prominently (Fig. 25). An M/16 KCl solution produces swelling and explosion of the spermatozoa (Fig. 26). In many instances the explosion is so violent that the nuclear cup ruptures completely.

6. *Potassium Nitrate.*—An M/2 KNO_3 solution which is isotonic with sea water does not produce any explosion. However, the protoplasmic cup swells and becomes more homogeneous in appearance. Also the primary and secondary vesicles become more distinctly marked off from each other (Fig. 27). An M/4 KNO_3 mixture has a similar effect. An M/8 KNO_3 solution brings about a swelling of the protoplasmic cup and a slow eversion of the second vesicle so that the spermatozoa resemble Fig. 28. In an M/16 KNO_3 solution the spermatozoa explode very rapidly and they come to look like Fig. 26.

7. *Potassium Hydroxide.*—Very dilute solutions of KOH bring forth a violent reaction in the spermatozoa. The protoplasmic cup swells, becomes homogeneous and at the same time pushes the vesicles upward (Figs. 29–31). The secondary vesicle undergoes a rotation and is pushed to one side. Finally the vesicles explode with great violence and the entire spermatozoön soon goes to pieces.

8. *Distilled Water.*—Distilled water produces a rapid eversion of the vesicles so that in a very short time the spermatozoa come to resemble Figs. 32 and 33.

9. *Cane Sugar.*—From the above experiments two conclusions might be inferred regarding the explosion of the spermatozoa, one is that it is due to lack of electrolytes, and the other is that the explosion is due to a reduction of the osmotic pressure produced by surrounding the spermatozoa with a hypotonic solution. In order to determine which factor we have to deal with, the spermatozoa were surrounded with an M/1 cane sugar solution which is approximately isotonic with sea water. If the factor involved were due to lack of electrolytes then, since the sugar solution contains no electrolytes, the spermatozoa ought to explode. But the M/1 cane sugar solution did not produce any

change in the normal appearance of the spermatozoa, thereby pointing to the second factor, namely, osmotic pressure, as the one which undoubtedly operates in bringing about the eversion of the vesicles.

10. *Ovarian Fluid.*—Since a reduction in osmotic pressure produces the explosion of the spermatozoa, the next question which naturally arises is whether the female gonads at the time of fertilization produce a hypotonic substance which, when coming in contact with the spermatozoa, causes them to explode, thereby bringing about fertilization of the ova. In order to test this out, the ovaries and oviducts were mashed up in sea water and the living spermatozoa were then surrounded by this mixture. In some cases (not all), a few of the spermatozoa exploded violently. The nuclear cup at first swelled and became homogeneous (Fig. 34). Then the vesicles were everted with considerable force and in many instances, the primary vesicle, or both the primary and secondary vesicles completely disintegrated, leaving stages like those shown in Figs. 35–39. Whether this was due to some agent produced by the female gonads or to some other agent cannot be definitely stated, for not all of the spermatozoa were affected in the same manner as those mentioned above. However, it is also significant that the ovaries used during the months of the year when the investigations were conducted (June and July), were past maturity. They were small and immature and this might account for the results obtained. Another significant fact to be taken into consideration is that in control experiments in which living spermatozoa from the same males as those used in the experiments with the ovarian fluids, were surrounded with sea water alone, none of the spermatozoa exploded. Now, the question arises, why should we get a violent explosion of even a few spermatozoa when ovarian contents are used and no explosion when the ovarian fluids are lacking? I am strongly of the opinion that the female gonads produce some substance which is responsible for the explosion. Also, it seems very probable that at the time of sexual maturity of the female this specific substance must be present in such quantities as to activate all of the living spermatozoa.

11. *Acidulated Sea Water.*—In all cases weak dilutions of the

acids were used. If the acid was a liquid, the dilution used was 1 part of the concentrated acid dissolved in 25 parts of sea water. A drop of this was then added to the edge of the cover glass under which the living spermatozoa were held suspended in sea water. If the acid used was crystalline in texture, then a few of the crystals were placed at the edge of the cover glass and allowed to dissolve slowly under it.

(*a*) *Glacial Acetic Acid.*—Causes the protoplasmic cups to lose their granular and vacuolated appearance. Usually two or three dark granules remain in the nuclear cup. The nuclear cups and radial arms swell and lose their color (Figs. 40 and 41). The spermatozoa in many instances are thrown together into aggregates (Fig. 42). After remaining exposed to the action of the acid for some time many of the spermatozoa explode (Fig. 43) and disintegrate completely.

(*b*) *Salicylic Acid.*—Reaction here is similar to that caused by glacial acetic acid.

(*c*) *Saponin.*—Causes considerable swelling (Fig. 44). Nuclear cup and radial arms become more homogeneous and much paler in color. They appear almost transparent. A few of the spermatozoa explode after being exposed for some time.

(*d*) *Sodium Glycocholate.*—Causes swelling similar to that produced by saponin or glacial acetic acid. During this swelling the vacuoles of the nuclear cup at first enlarge and then disappear, giving the nuclear cup a homogeneous appearance. Soon a violent explosion of vesicles takes place. Nuclear cup now loses its greenish color, becomes ragged and transparent with small dark spots. Very shortly the spermatozoa disintegrate.

(*e*) *Nitric Acid.*—This brings about a homogeneity of appearance in protoplasmic cup with considerable shrinkage (Fig. 45). The second vesicle in many cases is everted (Fig. 46).

(*f*) *Hydrochloric Acid.*—The reaction here is very similar to that caused by nitric acid.

(*g*) *Oxalic Acid.*—Reaction is similar to that produced by nitric acid.

(*h*) *Tannic Acid.*—Reaction is similar to that of nitric acid, with the exception that none of the vesicles are everted.

(*i*) *Picric Acid.*—The reaction produced in the spermatozoa

is the same as that brought about by tannic acid. The spermatozoa are soon killed and stained a yellowish-green.

(*j*) *Chromic Acid.*—This produces a similar result to that obtained with either tannic or picric acids. Here the spermatozoa are fixed a yellowish-brown.

Discussion.

A careful examination of the data presented in this paper shows quite clearly that a lowering of the osmotic pressure in the medium which surrounds the spermatozoa is responsible for their explosion. In this respect the present research bears out what Koltzoff ('06) first suggested for the explosion of the spermatozoa of other decapods. Also, Binford ('13) in *Menippe mercenaria* and the present writer in *Cambarus virilis* (Fasten, '14), and *Cancer magister* (Fasten, '18), have found that osmotic pressure accounts for the explosion of the spermatozoa. In the light of all this accumulated evidence it seems quite certain that the stimulating agent which brings about the explosion in the spermatozoa of the Decapoda, is one which reduces the osmotic pressure in the medium that surrounds them.

Since this is the operating factor, the question which naturally suggests itself is where in the Decapoda is such a stimulating agent produced? The writer is strongly of the opinion that the mature gonads of the female decapod produce some chemical substance which, when it comes in contact with the spermatozoa, brings about their explosion. The experiments with the ovarian fluids seem to point to such a conclusion. The work of Koltzoff ('06) and Binford ('13) also suggests a similar conclusion.

Concerning the function of the explosion, it, undoubtedly, acts as the force or the motive power which drives the spermatozoön into the egg during the process of fertilization. What parts of the spermatozoön actually penetrate the ovum during fertilization is still a debated question. Koltzoff ('06) and Spitschakoff ('09) are in agreement that the nuclear cup (derived from the nucleus of the spermatid) is the only structure which enters the ovum. Binford ('13), on the other hand, claims that the everted vesicles (cytoplasmic structures) of the exploded spermatozoön are driven into the egg, whereas the nuclear cup

remains on the outside where it soon disintegrates. In order to bring this mode of fertilization in harmony with the idea of the continuity of the chromosomes, Binford suggests that "the contents of the capsule (vesicles) may be derived from the nucleus of the spermatid and is probably oxychromatin which deposits basichromatin after it enters the egg and so gives rise to the chromosomes in the male pronucleus."

It is thus obvious that more research along this line is essential before any definite conclusions regarding fertilization in the Decapoda can be formulated. If we accept Binford's results then we must admit that they are contrary to everything that we know regarding fertilization in animals.

Summary.

1. The spermatozoa of the black-clawed crab, *Lophopanopeus bellus* (Stimpson) Rathbun, are minute, greenish cells, which appear like spheroids when seen from the top or bottom and like ellipsoids when seen from the side.

2. The structure of these spermatozoa is very similar to that of the edible crab, *Cancer magister* Dana. In the centre there is a tube-like central body, and surrounding this in order of sequence is a secondary vesicle, a primary vesicle and a nuclear cup with slender radiating arms.

3. There are four types of spermatozoa produced in *Lophopanopeus bellus*, depending on the number of radial arms which they contain. There are three-, four-, five- and six-rayed spermatozoa, with the four- and five-rayed types predominating in numbers.

4. In sea water and isotonic solutions of various salts, no change occurs in the normal appearance of the spermatozoa. In hypotonic solutions of these salts the spermatozoa explode by an eversion of the two vesicles and the central body.

5. In ovarian fluids some of the spermatozoa explode violently, with a rupture and disintegration of one or both vesicles.

6. Acidulated sea water has a harmful effect on the spermatozoa, either causing swelling or shrinkage, with subsequent disintegration.

7. A lowering of the osmotic pressure in the medium that

surrounds the spermatozoa, undoubtedly brings about their explosion.

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DESCRIPTION OF PLATES.

All figures in the accompanying plates were made with the aid of the camera lucida. All figures, except Figs. 3–7, were made from living spermatozoa. Figs 3–7 are drawings of spermatozoa which were fixed by osmic acid fumes and stained with Heidenhain's iron-hæmatoxylin and acid-fuchsin. The magnification of Figs. 1–7 is 3,300 times; that of Figs. 8–33 is 1,400 times, and that of Figs. 34–46. is 1,700 times.

Explanation of Plate I.

Figs. 1 and 2. Bottom and side views of living spermatozoa suspended in crab's body fluid. *b*, central body; *h*, protoplasmic cup; *v*, primary vesicle; *v'*, secondary vesicle.

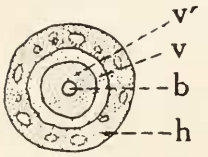
Fig. 3. Side view of spermatozoön fixed in osmic acid fumes and stained with iron-hæmatoxylin and acid-fuchsin. *b*, central body; *n*, nuclear cup; *r*, radial arms; *v*, primary vesicle; *v'*, secondary vesicle.

Figs. 4 to 7. Bottom views of spermatozoa fixed in osmic acid fumes and stained with iron-hæmatoxylin and acid-fuchsin, showing, respectively, three-, four-, five- and six-rayed types.

Figs. 8 and 9. Spermatozoa in sea water.

Figs. 10 to 19. Spermatozoa in various concentrations of sodium chloride.

Figs. 20 to 23. Spermatozoa in various concentrations of calcium chloride.



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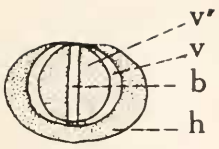

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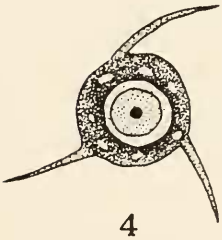

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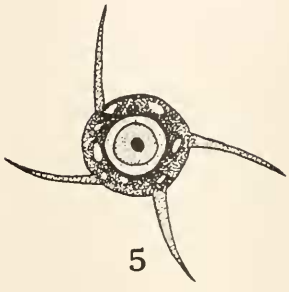

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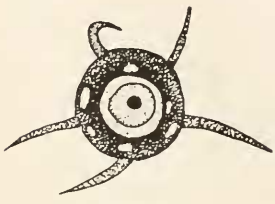

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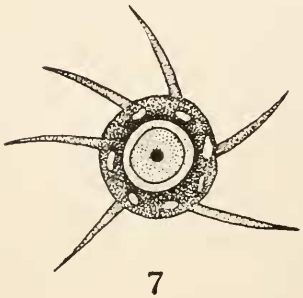

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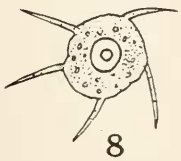

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NATHAN FASTEN.

Explanation of Plate II.

Fig. 24. Spermatozoön which has exploded in a hypotonic solution of calcium chloride.

Figs. 25 to 28. Spermatozoa which have been exposed to various concentrations of potassium chloride.

Figs. 29 to 31. Spermatozoa in weak solutions of potassium hydroxide.

Figs. 32 and 33. Spermatozoa which have exploded in distilled water.

Figs. 34 to 39. Spermatozoa which have been subjected to the effects of ovarian fluids.

Figs. 40 to 43. Spermatozoa which have been acted on by glacial acetic acid in sea water.

Fig. 44. Bottom view of spermatozoön which has been exposed to saponin in sea water.

Figs. 45 and 46. Spermatozoa which have been acted on by nitric acid in sea water.

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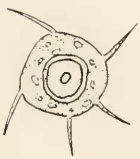

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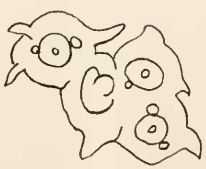

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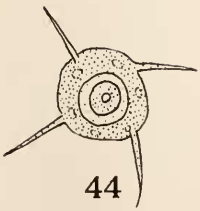

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NATHAN FASTEN.

A LEAF MIMICKING FISH.[1]

CARL H. EIGENMANN AND WILLIAM RAY ALLEN.

Heckel, in Johann Natterer's "Neue Flussfische Brasilien's nach den Beobachtungen und Mittheilungen des Entdeckers,"[2] described a small fish 3.5 inches long from a forest pool along the Rio Negro. He named it *Monocirrhus polyacanthus* and stated that at Marabitanas it was called *pirá-cáa* which means leaf-fish. Marabitanas is less than one degree north of the equator near the fiftieth degree of west latitude, in other words about sixty miles south of the southern end of the Rio Cassiquiare. No other specimens have been recorded. Günther placed it with *Polycentrus* to constitute the family Polycentridæ. The leaf-like appearance evidently impressed the Indians about Marabitanas who were acquainted with it and had a name for it.

During the Centennial Expedition of Indiana University, Dr. Allen secured three specimens of the same, or of a similar species from a brook near the Rio Itaya at Iquitos on the Peruvian Amazon.

The junior author reports that this fish was collected on September 19, 1920, while a guest of Don Antonio Layet at his hacienda, about six kilometers up the Rio Itaya from Iquitos. It was found in a small, sluggish brook which flows over very flat second bottom land, seldom inundated, and in the midst of dense forest.

There had not been much recent local rain, and there was only a slightly perceptible trickle of current at the riffles. Most of the brook was now reduced to quiet pools ten to fifteen feet across, densely overhung by vegetation, and shaded except for an hour or two at mid-day. The water was clear and of a slightly brown color, the bottom brushy, and matted with fallen leaves.

"Sr. Layet's servants had just introduced poison for me at the riffles, allowing it to flow slowly into the pools. Others with their long knives had made paths by which the low bank could

[1] Contribution from the Zoölogical Laboratory of Indiana University, No. 183.

[2] Ann. Wien Mus., 1840, II., p. 439.

be followed, The poison used was the milky sap washed from the pounded roots of *cube* (or *barbasco*), a plant cultivated as a fish poison and insecticide wash for cattle.

"I was beginning to grow impatient at the slowness of the poison, and to wonder if our long wait was going to be useless. I had observed several different species of fishes but they did not seem to be yielding to the usual respiratory difficulties following *cube*-poisoning, nor even to be trying to escape past the seines which we had stretched across the brook above and below.

"In order to know if there was sufficient current to carry the poison to every part of the pool, I began tossing broken twigs on the water to observe their course with the current. One such twig had reached a standstill, when directly beneath it I saw what was apparently a dead leaf being wafted past the twig. I couldn't understand why the twig was not moving too. At about that moment the leaf moved out into a path of sunlight, and toward the surface. There the resemblance to a fish became apparent, especially to one in search of the same. Its behavior, too, was like that of a poisoned fish struggling for oxygen."

The outline of the fish is similar to that of an asymmetrical leaf. The erected spinous dorsal and anal with their serrated character are not unlike the toothed edge of a leaf. The mimicry in color and markings is very close, the photograph and drawing of the dead specimens scarcely doing it justice. The lateral band has a position like that of a midrib of an asymmetric leaf. Like a midrib it fades away before reaching the distal margin. A petiole is not lacking, for the sharp, elongated snout and the protractile barbel carry out the resemblance.

While this fish may fall short of the perfection in mimicry exhibited by Kallima, it does take due account of the fact that few perfect leaves exsist, especially by the time they have reached the water. The transparent dorsal and soft anal between the spinous fins and caudal peduncle resemble breaks in the margin of a leaf. Futhermore the faded and discolored portions of many leaves, due to fungi, have their counterpart in the more ashy triangular area in the forward half of the fish.

The mimicry of *M. mimophyllus* has a physiological side. When swimming it moves in a gliding manner (like a seahorse)

that resembles a drifting leaf. This movement is due chiefly to the rapid beating of the small transparent soft dorsal and soft anal. These fins are set within the outline of the body, their bases being transverse to the body length. They have the direct forward push of the screws of a ship. Being hyaline their motion does not attract attention.

Several other species of fishes in the forest pools have the color of dead leaves. The others were seen before yielding to the poison, while *M. mimophyllus*, with a much more complete mimicry, was not.

A technical diagnosis of the species follows.

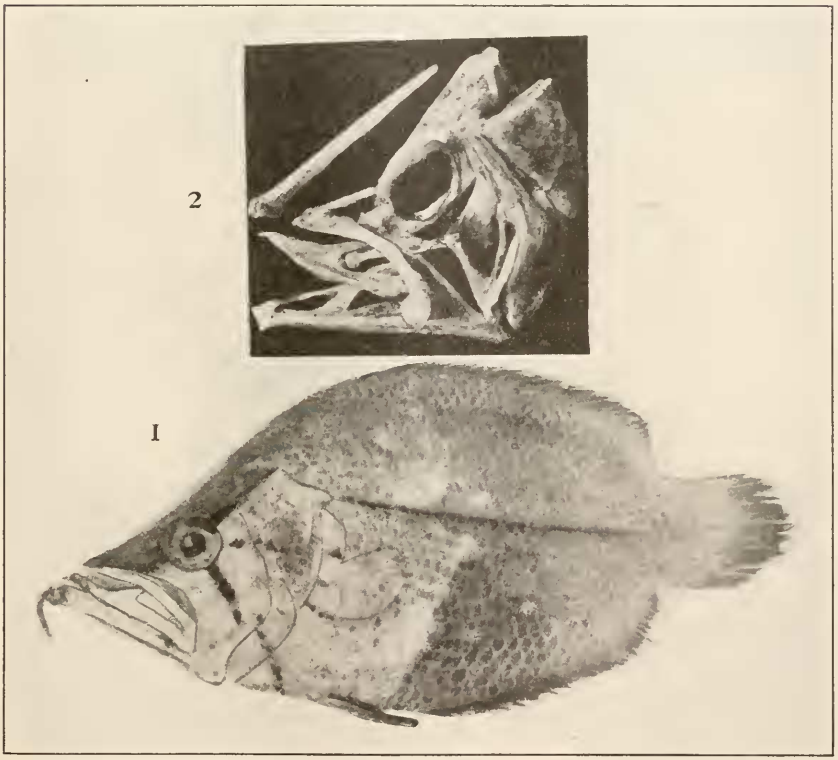

FIG. 1. Photograph of a specimen 61 mm. over all.

FIG. 2. Photograph of the skeleton of the head. The premaxillary spine is broken off from the rest of the bone, and the posterior end of the premaxillary has slipped upward a little away from its original position.

MONOCIRRHUS MIMOPHYLLUS Eigenmann & Allen spec. nov.

15715, I., 3, 44, 47, and 51 mm. long to base of caudal (65 mm. over all). Brooks near the Rio Itaya, Iquitos. Collected by Dr. W. R. Allen.

Evidently closely allied to *M. polyacanthus* Heckel, if distinct. In *M. polyacanthus* the caudal is said to be emarginate, the lateral band is said to run through the lower half of the tail, and the edge of the dorsal, anal, and tip of the ventrals are said to be blackish, the end of the caudal white.

Head 2.5; depth 1.92; D. XVI or XVII, 13; A. XII or XIII, 12 to 14.

Greatly compressed, the snout very sharp, the chin projecting, with a goatee barbel; the two rami of the mandible in contact below, equal in length to the head behind the anterior nares; maxillaries equal to snout and eye; premaxillaries greatly protractile; eye 1.5 in snout, 4 in the head, about .8 in the interorbital; opercular spine on a line between the upper margin of the orbit and the upper margin of the caudal peduncle. Profile between snout and occiput concave; gill-membranes somewhat united, entirely free from the isthmus, entirely hidden by the rami of the mandible.

Tongue very long and slender, rod-like, the free portion about as long as the eye, its tip soft, curved up and slightly cupped; premaxillary spine extending far beyond the eye, equal to the length of the mandible; mandible with one, in part two series of minute, recurved teeth; premaxillary with a single series of teeth on the sides, a triangular patch of teeth at the tip; no teeth on roof of mouth.

Pectoral broad, its length about 3 in the head, soft-rayed; distance between tip of the snout and origin of the dorsal a little more or a little less than 2 in the length without caudal; base of the spinous dorsal 2 in the length; base of soft dorsal about one-fifth of the length of the spinous dorsal; caudal *rounded*, equal to snout and eye or a little shorter; origin of anal and third dorsal spine equidistant from tip of snout; base of spinous portion of the anal about three in the length; base of soft part of anal a trifle longer than base of soft dorsal; ventrals reaching origin of anal, their inner ray adnate. First ray stout and spinous. Cheeks, opercle, and top of head to tip of snout scaled; preorbital

the only portion of the head naked. Scales of sides regularly imbricate, without lateral line pores; dorsal and anal partially depressible into a scaly sheath, the spines alternating when depressed. The scales of the sides roughened on half their exposed part, margined with very fine hyaline spinules.

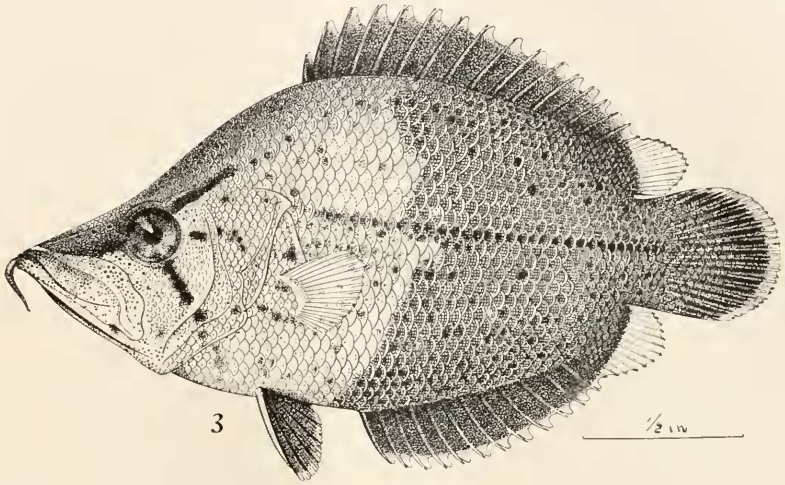

FIG. 3. Drawing of another specimen by W. S. Atkinson.

Four gill arches, lower arch of the first one with eleven rakers, the first a spinulous patch without projection, graduated to the last one which is about two thirds as long as the eye; all of them with numerous small spines; only two spinulous cushions on the upper arch; pharyngeal teeth similar to those on the gill-rakers.

Pectorals, soft dorsal, soft anal, margins of spinous dorsal, spinous anal and caudal and to a less extent of the ventrals hyaline; the hyaline of all but the soft dorsal, soft anal and pectoral bordered by black; a dark line from above the upper angle of the gill opening through the middle of the eye to the maxillary, a similar line from the eye through the cheeks crossing the breast half way between the ventrals and the gill opening, another one extending straight back from the eye; a similar dark line extending from the point of the opercle to the middle of the caudal peduncle; area from the middle of the ventrals up to the dorsal, and then forward below the line through the eye to the mandible several shades lighter than the back or the area behind this line. Slightly coppery color in living fish, this shade lost in alcohol.

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BIOLOGICAL BULLETIN

THE EFFECT OF IODINE AND IODOTHYRINE ON THE LARVÆ OF SALAMANDERS. II. THE RELATION BETWEEN METAMORPHOSIS AND LIMB DEVELOPMENT IN SALAMANDER LARVÆ.

E. UHLENHUTH.

(From the Laboratories of the Rockefeller Institute for Medical Research.)

In previous experiments (1) on the larvæ of *Ambystoma opacum* I found that iodothyrine did not accelerate the development of the limbs, although it caused rapid metamorphosis. Consequently, if the administration of iodothyrine was begun at an early larval stage, the metamorphosed salamanders possessed hind limbs which did not have the full number of toes. In agreement with these observations is the fact that feeding of thymus gland, although it resulted in an inhibition of metamorphosis, did not retard the development of the limbs of the thymus-fed salamander larvæ (2). Hence it is evident that in salamander larvæ the development of the limbs is independent of the substance (thyroid hormone) which causes metamorphosis.

The relation between limb development and metamorphosis as it exists in salamander larvæ is of especial interest, since apparently it is just the opposite of what should have been expected from the experiments performed on the anuran tadpoles. Through the work of Gudernatsch (3) and many other investigators it is well known that in tadpoles administration of thyroid gland, iodothyrine and other thyroid preparations accelerates not only metamorphosis, but also the development of the limbs. Lately Swingle (4) found that the administration of inorganic iodine which causes precocious metamorphosis of the tadpoles likewise accelerates development of the limbs.

Recent investigations have shown that the thyroid hormone and probably other morphogenic hormones, by increasing the rate of certain fundamental reactions, have the ability of causing structural changes throughout the entire organism, bringing thus about morphological expressions of a wide range affecting nearly the whole body. It seems that these hormonic substances, as far as their immediate effect is concerned, act chiefly by inducing a general histolysis throughout the various organs of the organism. There can be no doubt, however, that, besides these hormones referred to above, other substances play an important rôle in the development of an organism; these substances seem to possess a more localized action, effect the development of only certain organs and are concerned chiefly with the building up of the structures of these organs. Certainly, in the evolution of the organisms, the acquirement of the ability of elaborating the latter kind of substances must have played a rôle equally important as that played by substances such as the thyroid hormone. The limbs of the amphibians are apparently organs whose development seems to depend chiefly on the action of substances belonging to the latter group of substances and not on the activity of the thyroid hormone.

On account of the increased importance which, in the light of such considerations, seemed to attach itself to the finding that the development of the salamander limbs is independent of the thyroid hormone, it appeared necessary to repeat my previous experiments on the relation between limb development and metamorphosis. The present article will be devoted to reporting these new experiments. They consisted in causing precocious metamorphosis of the larvæ of *Ambystoma maculatum* by keeping them in iodothyrine, whereby special attention was paid to a possible acceleration of limb development. The result was the same as in the experiments on *A. opacum;* the rate of the development of the limbs remained unchanged, although metamorphosis took place at an early date. Not only larvæ, but also embryos, at early stages, were exposed to the influence of iodothyrine, in order to avoid the objection that failure of the iodothyrine to cause accelerated limb development was due to the experiments having been started at a stage at which limb development was too far advanced. Again

these experiments were completely negative as to an acceleration of limb development. In order to be certain that the method employed in my experiments on salamanders was correct, several tests were carried out on tadpoles; these were positive.

In addition to these experiments, several experiments were made to test the influence of inorganic iodine on the development of the amphibian limbs. Like Swingle (4), I found a distinct acceleration of the limb development in tadpoles; in the salamander larvæ, however, iodine had no effect on the development of the limbs. The bearing of this result, which is different from that of the experiments with iodothyrine will be referred to in the discussion.

Experiments on Tadpoles.

The experiments on tadpoles, which were intended to serve as a check to the experiments on salamanders, were not advanced beyond a merely preliminary stage, since they gave positive results from the very beginning. As they are confirmative of the observations reported by other investigators, they will be reported only briefly.

In one experiment on the larvæ of *Rana sylvatica* the controls were kept under observation till the 66th day after hatching. At this time the hind limbs of the control larva furthest advanced possessed 3 distinctly differentiated toes, while the 2 other toes were still rudimentary. In the iodothyrine series (kept from the 18th to the 26th day in water which contained 0.005 gm. Bayer's iodothyrine per 1,000 c.c. of water) the fore limbs broke through the walls of the gill chamber on the 33d day after hatching in every one of the 3 larvæ surviving, at this date, from the 6 larvæ composing the series at the beginning of the experiment. The inorganic iodine, in the concentration used in this experiment (2 to 3 drops of a 1/20 M solution of iodine per 1,000 c.c. of water), as well as in other experiments, proved to be considerably less effective than the iodothyrine, as 66 days after hatching the fore limbs had not perforated the gill chamber in a single instance. Yet the limbs, the hind limbs as well as the fore limbs, were distinctly further differentiated than in the controls of the same age; the hind limbs possessed 5 fully differentiated toes and in shape

were much like the hind limbs of an adult frog. Moreover, in 2 larvæ of the iodine series, which died at an age of 50 and 56 days respectively, the hind limbs possessed already at that date 5 fully differentiated toes.

In another experiment 5 series of the tadpoles of *Rana sylvatica* were kept in different concentrations of iodine (varying from 1 to 10 drops of a 1/20 M solution of iodine per 1,000 c.c. of water). The controls were kept under observation for 83 days; at the end of this period the hind limbs had remained undifferentiated, whitish buds in 5 of the 8 larvæ, while in the other 3 larvæ differentiation had taken place, the best differentiation being represented by 4 toes on the foot of the hind limbs. Many of the larvæ kept in inorganic iodine died; none of these was further advanced than the surviving larvæ. Among the surviving larvæ none had metamorphosed at the termination of the experiment, nor had the fore limbs broken through in a single instance; yet the limbs were considerably further differentiated than in the controls. In one larva of the iodine series, at an age of 73 days, the foot of the hind limbs was differentiated into 5 toes; in another larva, at an age of only 59 days, the hind limbs possessed 5 toes, and the fore limbs, which could be seen vigorously moving in the gill chamber, had developed 3 toes.

There can be no doubt that iodothyrine when administered to tadpoles greatly accelerates development of the limbs. Inorganic iodine, although it seemed less efficient in these experiments than iodothyrine, likewise increases the rate of the development of the limbs.

Experiments on Salamander Larvæ.

As pointed out in the introduction, my previous experiments on the larvæ of *Ambystoma opacum*, in which the administration of iodothyrine did not accelerate development of the limbs, were open to the criticism that the administration of iodothyrine was started at a stage at which limb development was fairly advanced (the toes having begun to differentiate), and that for this reason the iodothyrine may have been incapable of accelerating limb development. Therefore it seemed necessary to start the experiment at a very early stage. Two experiments were carried out, both on the embryos of *Ambystoma maculatum*.

Experiment I.—One egg mass of *Ambystoma maculatum* was collected on April 18, 1920. The eggs were not only freed from the general mass of jelly, but also the individual egg envelopes were removed, in order to assure ready access of the iodothyrine and iodine to the developing embryos.

Beginning of the experiment: 28 embryos selected; 9 of them placed into iodine-free water (10,000 c.c. H_2O, 0.16 gm. Na_2CO_3, 0.04 gm. K_2CO_3, 0.4 gm. $MgSO_4$–$7H_2O$, 0.6 gm. $CaCl_2$), 9 into iodine (2 drops of a 1/20 M solution of inorganic iodine per 1,000 c.c. of iodine-free water) and 10 into iodothyrine (0.01 gm. Bayer's iodothyrine per 1,000 c.c. of iodine-free water). In all embryos the first four visceral arches are formed; the fore-limb rudiments not yet differentiated from the pronephridial protuberance; no hind limbs.

The concentration of the inorganic iodine was increased to 8 drops per 1,000 c.c. 5 days, decreased to 6 drops 7 days, and decreased to 4 drops 11 days after the beginning of the experiment.

Sixteen days after the beginning of the experiment: Neither the iodine nor the iodothyrine had produced any influence on the development of the limbs (see Table I.).

TABLE I.

EXPERIMENT I.: 16 DAYS AFTER BEGINNING OF EXPERIMENT.

	Total Number.	Development of Toes.		
		3.5	3.0	2.5
Water	8	7		1
Iodine	8	5	1	2
Iodothyrin	10	9		1

Twenty days after the beginning of the experiment: The concentration of the iodine is decreased to 3 drops per 1,000 c.c. of water, the concentration of the iodothyrine increased to 0.1 gm. per 1,000 c.c. of water.

Twenty-seven days after the beginning of the experiment: Hind limbs commenced to differentiate into toes; but neither iodothyrine nor iodine accelerated limb development as compared to limb development of controls kept in iodine-free water (see Table II.).

Table II.

Experiment I.: 27 Days after Beginning of Experiment.

	Total Number.	Development of Toes.		
		6.0	4.5	4.0
Water	8	3	5	
Iodine	7		4	3
Iodothyrin	10		2	8

Yet the influence of the iodothyrine on metamorphosis had become noticeable in spite of the early stage of the larvæ, as the gills as well as the fin of the tail are found to be greatly atrophied.

Experiment II.—In this experiment one series of the embryos of *Ambystoma maculatum* was kept in inorganic iodine and one in iodine-free water. The concentration of iodine was 1 drop of a 1/20 M iodine solution per 1,000 c.c. of iodine-free water in the beginning, was increased later on to 8 drops and then gradually decreased to 3 drops. The embryos were at an early stage (formation of neural folds), when the experiment started; only the common jelly mass was removed.

Table III.

Experiment II.: 10 Days after Beginning of Experiment.

	Total Number.	Fore Limb Buds Present, No Indication of Toes.
Water	15	15
Iodine	14	14

Table IV.

Experiment II.: 18 Days after Beginning of Experiment.

	Total Number.[1]	Development of Toes.	
		3.5	2.5
Water	14	14	
Iodine	13	7	6

[1] Several eggs and embryos were attacked by moulds and as they disintegrated or developed abnormally, they had to be discarded causing thus the decreases in the total numbers.

TABLE V.

EXPERIMENT II.: 30 DAYS AFTER BEGINNING OF EXPERIMENT.

	Total Number.[1]	Development of Toes.		
		6.0	4.5	4.0
Water	14	5	7	2
Iodine	12	1	10	1

As Tables III., IV. and V. show, the inorganic iodine had no influence whatsoever on the development of the limbs.

The relation between limb development and metamorphosis was further tested in two experiments in which larvæ of *Ambystoma maculatum* at a more advanced stage were employed. Concerning the action of iodothyrine, the results were in complete accordance with those obtained in the larvæ of *Ambystoma opacum;* rapid metamorphosis, but no influence on limb development was observed. In each experiment one series was devoted to the study of the influence of inorganic iodine; this substance likewise had no influence on limb development, but unlike iodothyrine it did not cause precocious metamorphosis. Both iodine experiments as regards the influence of iodine on metamorphosis were described in detail in a previous article (5); they will be only briefly reported in this article.

In Experiment III. the larvæ were placed into iodine-free water containing 0.1 gm. iodothyrine per 1,000 c.c. of water at an age of 20 days, at which date nearly all larvæ had developed 4 toes in the fore limbs and several had commenced to develop the first 2 toes in the hind limbs. Thirty-three days after hatching—*i.e.,* 13 days after the first administration of iodothyrine—every one larva metamorphosed (as compared to 101 days in the controls), but in none of them the number of toes was more than 7.5, and in one it was only 6.0, this stage of limb development corresponding to the control series kept in iodine-free water without the addition of iodothyrine.

In Experiment IV. a smaller dosis of iodothyrine (0.01 gm. per 1,000 c.c. of iodine-free water) was administered. Precocious metamorphosis was caused also by this dosis, but the development of the limbs again remained completely unaffected as compared to

the controls. Since, however, metamorphosis took place at an age at which normally the limbs are fully developed (as shown by the controls), the precociously metamorphosed salamanders possessed in this experiment fully developed limbs.

Concerning the influence of inorganic iodine in these experiments, it was shown in a previous article (5) that administration of iodine does not result in precocious metamorphosis of salamander larvæ. In this article it should be added that it did have no effect also on the development of the limbs.

Discussion.

The experiments reported in this article confirm fully the observations made in my previous experiments. In the larvæ of salamanders the development of the limbs can not be accelerated by the administration of iodothyrine. Therefore, if iodothyrine is administered in doses which cause metamorphosis before the time at which, under normal conditions, the limbs are fully developed, metamorphosis takes place before the completion of limb development.

These facts demonstrate that in salamanders limb development is independent of the substance (thyroid hormone) which causes metamorphosis. This conclusion has recently been supported by several other facts. *Typhlomolge rathbuni,* the Texan cave salamander, does not possess a thyroid gland (6) and consequently does not metamorphose; yet its limbs develop in a normal manner. Hoskins and Hoskins (7) have shown that in the larvæ of *Ambystoma* the limbs develop normally, if the larvæ are deprived of their thyroid glands in early embryonic stages. This season I have repeated these experiments. Larvæ of *Ambystoma maculatum* were thyroidectomized at an early embryonic stage; these larvæ which are believed to have been successfully operated on (histological examination has not been made as yet) showed the same rate of limb development as the controls. Several larvæ of *Ambystoma tigrinum* were thyroidectomized at a stage at which 3 toes of the hind limbs were developed; the two other toes developed at a normal rate after thyroidectomy. These facts prove that in salamanders the substances causing limb development are not identical with those causing metamorphosis and consequently are not identical with the thyroid hormone.

In tadpoles substances which cause precocious metamorphosis also accelerate the development of the limbs. I have suggested, in a previous article (8), that in spite of this external difference existing between the larvæ of *Anura* and *Urodela* the primary effect of the thyroid hormone may be the same in both groups of animals, and the difference may not be a fundamentally different reaction upon the thyroid hormone, but merely a different mode of limb development.

There is no doubt that, except for the development of the limbs in tadpoles, the immediate effect of the thyroid hormone is, in both groups, predominantly a breaking down of tissues throughout the whole organism, not a building up of new organs. It is possible that in tadpoles the same substances endowed with a merely localized action cause limb development as in salamanders, but that in tadpoles these substances can not commence to build up the structures of the limbs before some obstacle has been cleared away by the action of the thyroid hormone. That the thyroid hormone controls limb development in the tadpoles does not necessarily mean that it has any part in the constructive processes of limb development. If we consider the advanced stages of the development of the fore limbs in tadpoles, we find conditions which make it indeed very probable that the thyroid hormone, in this process, plays merely the rôle of removing an obstacle external to the tissues of the limb itself. In salamanders both hind and fore limbs develop freely, while in tadpoles the fore limbs are inclosed in the gill chamber. In order that they may break through the walls of the gill chamber, certain changes of the skin and the tissues underlying it must take place; these changes are not caused by the legs themselves, but take place even in the absence of the limbs (9) at the time at which metamorphosis occurs. I have pointed out repeatedly that in salamanders one of the most conspicuous effects of the thyroid hormone is a certain change of the skin which finally results in the shedding of the skin and may be identical with the process which leads to the atrophy of the gills. A similar change is brought about in the skin of the tadpoles; in the tadpoles, too, the skin is shed for the first time when metamorphosis takes place. It is possible that the change of the skin which is necessary to

permit of the freeing of the fore limbs is identical with the change that causes the first shedding of the skin.

In support of this view is the fact that the freeing of the fore limbs is clearly the only step in the development of the limbs, which in not one single instance has been observed to take place in tadpoles which had been deprived of the thyroid secretion, while the developmental processes preceding the freeing of the limb may take place in thyroidectomized tadpoles. Allen (10) states that in tadpoles of *Bufo*, in the complete absence of a thyroid, both fore and hind limbs develop normally (and even grow larger than the limbs of normal larvæ)—*i.e.*, behave exactly like the limbs of salamander larvæ—yet the fore limbs fail to break through the walls of the gill chamber. Apparently in this anuran species the development of the limbs, except for the freeing of the fore limbs, is independent of the thyroid hormone as it is in the urodelan larvæ.

Should these views prove to be correct, it would seem probable that the mode of limb development in amphibians is the morphologic expression of the existence of two kinds of morphogenic substances; one group of these substances serves to procure the actual building stones of the morphological structures of the organ, while the other group of substances merely brings about a general histolysis of old structures, removing thus obstacles to the action of the substances belonging to the first group of substances.

As has been mentioned above, the ineffectiveness of inorganic iodine in the limb development of salamanders has a reason different from that of the ineffectiveness of iodothyrine. In a previous article (5) I have shown that, in contradistinction to iodothyrine, the administration of inorganic iodine does not produce precocious metamorphosis in salamander larvæ. The inorganic iodine has no effect on the salamander metamorphosis, because the thyroid hormone in salamanders is not released during the greater part of the larval period, and a greater supply of inorganic iodine, even if it should result in the elaboration of an excess of thyroid hormone, as it actually does in tadpoles (11), can not make itself felt in the salamander larvæ in consequence of the retention of the hormone. As has been shown in this article, inorganic iodine, like iodothyrine, has no effect on limb development of salamanders. But it must

be borne in mind that the ineffectiveness of inorganic iodine in limb development of salamanders is merely due to the above-mentioned peculiarity of the thyroid apparatus of the salamander larvæ and not to the fact that limb development in salamanders is independent of the thyroid hormone and of metamorphosis. Inorganic iodine could not accelerate limb development of salamanders, even if iodothyrine would have an accelerating effect.

Summary.

1. Tadpoles of *Rana sylvatica* were kept in iodothyrine and in solutions of inorganic iodine. Both substances were found to accelerate limb development. This result confirms the observations of previous investigations.

2. Embryos as well as larvæ, in early stages, of *Ambystoma maculatum* were kept in iodothyrine and in inorganic iodine. Neither of these substances accelerated the development of the limbs, although iodothyrine caused rapid metamorphosis of the salamander larvæ.

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MICRODISSECTION STUDIES, III. SOME PROBLEMS IN THE MATURATION AND FERTILIZATION OF THE ECHINODERM EGG.

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This paper is a record of operative work on the starfish, sea-urchin and sand-dollar eggs to ascertain the morphological nature of changes which take place in the egg during its maturation and fertilization. Results were obtained on the effect of nuclear material on cytoplasm, the nature of cortical changes in the maturing and fertilized egg and the difference between cortex and medulla of the egg with respect to fertilizability and to other life activities. The dissection and injection of the living eggs were carried out at first by means of Barber's ('14) apparatus and later with an improved micromanipulator of my own design ('21[b]). A description of the technique as applied to microdissection has already been published (Chambers, '18[a]). A detailed description of the new micromanipulator will appear both in the *Journal of Bacteriology* and in the *Anatomical Record*.

1. THE GERMINAL VESICLE IN THE MATURING STARFISH EGG.

Starfish eggs, on being shed naturally, have already begun maturing. In order, however, to secure large quantities of eggs, it has been the general custom to remove the ovaries bodily from a ripe female and to cut them up in a bowl of sea water. This procedure brings the eggs into the sea water in the immature condition with germinal vesicles intact. The germinal vesicle begins to disappear anywhere from thirty to fifty minutes after the eggs come into contact with the sea water and maturation usually proceeds in a normal manner (Wilson and Mathews, '95).

The undisturbed germinal vesicle or nucleus of a fully grown

immature egg is a hyaline sphere containing a sharply differentiated nucleolus and occupying about one fifth the volume of the egg. With the microdissection needle the vesicle may be moved about in the fluid cytoplasm without injury to the egg. With the needle one may considerably indent the surface of the vesicle. On removal of the needle the vesicle reverts again to the spherical shape (Fig. 1). The vesicle possesses a morphologically definite surface membrane inclosing an optically homogeneous liquid (cf. Chambers, '18[b]). Within this liquid lies a visible body, the nucleolus. By agitating the vesicle the nucleolus may be made to occupy any position within the nuclear fluid. The nuclear membrane is very easily injured. If, however, a microneedle be carefully inserted into the nucleus, the membrane about the puncture adheres to the body of the needle and the tip of the needle may push the nucleolus about with no apparent injury. The existence of considerable tension in the nuclear membrane is shown in the following experiment. An egg was cut into three fragments in such a way that the surface film forming over the cut surfaces of the middle fragment pressed upon the nucleus, deforming it considerably (Fig. 2). The attempt of the nucleus to return to a spherical shape bulged out one end of the egg fragment until it was constricted off from the remainder of the fragment (Fig. 2*b–f*).

Tearing the nuclear membrane in most cases results in a destruction of the nucleus. In a few cases it was possible to produce a slight rupture with no noticeable injurious effects. Such a case is recorded in Fig. 3. At 10:44 A.M. undue pressure on the germinal vesicle when cutting an immature egg in two resulted in its rupture followed by a lobular extrusion bounded by a very delicate film. During the following ten minutes the vesicle began slowly to revert to its original shape (Fig. 3*b* and *c*). Before that was attained the maturation process began and, at 10:55, the outline of the vesicle had disappeared. The nucleated egg fragment maturated normally and five hours and a half after insemination it had segmented in two. At 8:40 P.M. it had developed into a swimming blastula.

The cytoplasm of the egg allows of considerable tearing without

apparent injury (Chambers, '17-*a*). If, however, the nuclear membrane be torn, a very striking phenomenon occurs. The cytoplasm immediately surrounding the nucleus disintegrates and

Fig. 1

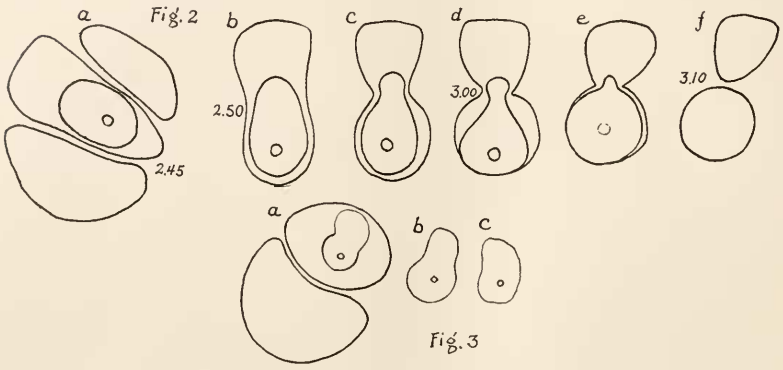

Fig. 1. Figures showing the extent to which the nucleus (germinal vesicle) of an immature starfish egg may be indented on one or both sides without rupture. On removing the needle the nucleus reverts to its original spherical shape.

Fig. 2. *a*, immature starfish egg cut at 2:45 P.M. into three parts; the nucleus has remained intact but is laterally compressed in the middle fragment. *b*, *c*, *d*, *e* and *f*, successive steps in attempt of nucleus to round up; *b*, 2:50 P.M.; *d*, 3:00 P.M.; *f*, 3:10 P.M.

Fig. 3. *a*, partial rupture of nucleus followed by a repair of its membrane. *b* and *c*, successive changes in the shape of the nucleus within the following ten minutes after which time it disappeared.

liquefies. If the rupture of the nucleus be violent, the disintegration of the cytoplasm spreads rapidly until the entire egg is involved. If the rupture be slight, the disintegrative process is quickly limited by a surface film which forms on the boundary between the disintegrating and the surrounding healthy cytoplasm (Fig. 4). This film tends to prevent any further spread of the destructive process. The destruction of the cytoplasm is evidently due to something which emanates from the injured nucleus. The injury to the cytoplasm does not start where the nuclear membrane is first torn, but from the entire surface of the injured nucleus.

This is analogous to results obtained by injuring red blood corpuscles with a needle upon which hemoglobin escapes immediately from the entire surface (Chambers, '15).

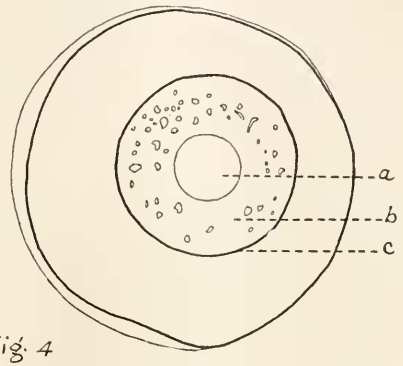

FIG. 4. Disintegration of cytoplasm surrounding the nucleus on tearing the nucleus with a needle. (*a*) Faint hyaline sphere, a remnant of the destroyed nucleus. (*b*) Disintegrated cytoplasm. (*c*) Cytoplasmic surface film separating disintegrated from healthy cytoplasm.

Within the nucleus itself the immediate effect of the injury is a dissolution of the nucleolus. A nuclear remnant tends to persist after the injury as a hyaline sphere lying within the disintegration products of the cytoplasm. On being touched with the needle it fades from view.

In permanently immature eggs, such as eggs which have been standing in sea water for an hour or more without maturing, the disintegrative effect on the cytoplasm by injuring the nucleus tends to be much more restricted, and the nuclear sphere which persists after the injury can be shown to possess a morphologically definite membrane. Such a sphere is easily dissected out of the egg. Frequently, when the germinal vesicle lies close to the periphery of the egg, the disintegration of the cytoplasm quickly reaches the surface. With the formation of a surface film over the healthy cytoplasm the disintegrative area lies in a deep bay on one side of the egg. This hollow is slowly obliterated as the semi-fluid substance of the egg strives to assume a spherical shape. In this way the disintegrated material is forced out of the egg together with the persisting nuclear sphere. This nuclear sphere persists for some time in the sea water. It can be deformed by means of the needle and, on

tearing its surface, the fluid contents escape, leaving behind a collapsed membrane which disappears within 10 to 15 seconds.

Fig. 5 shows the effect of cutting the mature egg nucleus of the starfish egg. By pushing the nucleus against the inner surface of

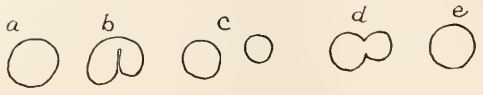

Fig. 5

FIG. 5. Effect of cutting mature nucleus of a starfish (*Asterias*) or sea-urchin (*Arbacia*) egg. *a*, intact egg nucleus; *b*, nucleus in process of being cut in two. The nucleus was pushed against the periphery of the egg as it was being cut by a vertical needle; *c*, the separated fragments of the nucleus; *d*, reunion of the fragments; *e*, reconstituted nucleus.

the egg it is possible to pinch it into two pieces. Each piece rounds up but, if the two are allowed to come into contact, they will fuse into a single nucleus again. The same result obtains in the sand-dollar and sea-urchin eggs. If, however, the nuclear membrane be torn, a disintegration of the cytoplasm results analogous to that produced on rupturing the germinal vesicle. The extent of disintegration is much more limited, owing doubtless to the much smaller amount of nuclear material present. Similar results were obtained on tearing the nucleus of the *Arbacia* egg.

It was found possible to destroy the cytoplasm of one egg by injecting into it nuclear material obtained from another egg. This experiment has to be performed very rapidly, for if the nuclear material be allowed to remain longer than ten seconds within the pipette it has no effect whatever when injected into the cytoplasm of an egg. If it be injected within that time the destructive effect is very pronounced.

If an egg be allowed to undergo normal maturation, the germinal vesicle disappears except for a small remnant which becomes the definite egg nucleus. This egg nucleus moves to the surface of the egg, where it gives off the two polar bodies. It then constitutes the female pronucleus, which remains quiescent until fertilization occurs. The disappearance of the germinal vesicle is a well-known phenomenon. In order, however, to locate definite stages selected for my operations I introduce the following sum-

mary. The germinal vesicle with an intact membrane is shown in Fig. 6. Within thirty to forty-five minutes after standing in sea water the nuclear membrane exhibits wrinkles and its outline begins to fade from view. Within a few minutes no membrane is visible and cytoplasmic granules can be seen moving into the region hitherto occupied by the nucleus, while the nuclear sap appears to be diffusing out (Fig. 6-*c*). As the nuclear membrane disappears the nucleolus fades from view. The invasion of the nuclear area by cytoplasmic granules continues until all of the area except a small portion is rendered indistinguishable from the general cytoplasm of the egg. This small portion persists as the egg nucleus (Fig 6*e* and *f*). In Fig. 6-*g* two consecutive positions of the nucleus are shown. At 1 : 13 P.M. it lay deep in the substance of the egg. In twenty minutes it had moved to the periphery of the egg preparatory to the formation of the polar bodies.

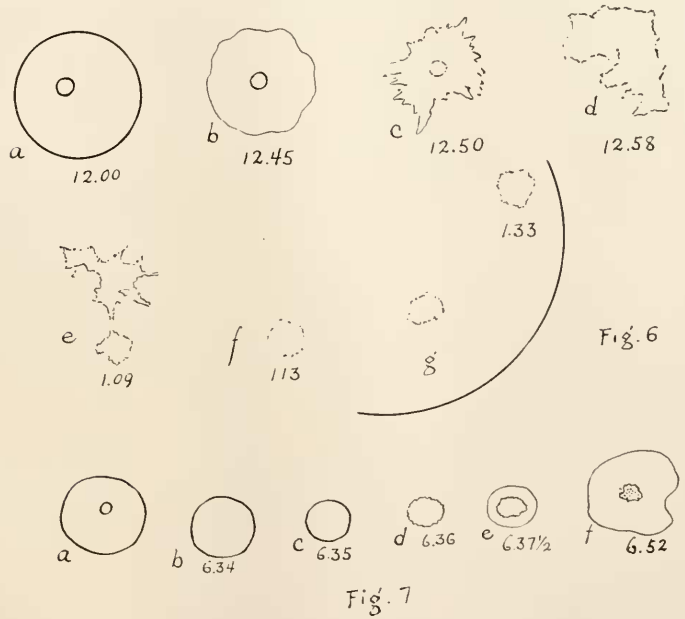

FIG. 6. Camera lucida drawings of the successive steps in the normal dissolution of the germinal vesicle in the maturing starfish egg. The process was somewhat slowed down owing possibly to the compressed condition of the egg necessary for detailed observation.

FIG. 7. *a*, intact germinal vesicle within the egg. *b*, nucleus after having been torn out of the egg and brought into sea water. *c*, *d*, *e* and *f*, successive changes undergone by the nucleus lying in sea water.

By means of the microdissection needle it is possible to show, at the stage shown in Fig. 6-*d*, that the membrane of the germinal vesicle no longer exists. By careful manipulation it was possible to push the cytoplasmic granules into the nuclear area. A slight rapid movement of the needle, however, was sufficient to give rise to disintegrative processes similar to those on tearing an intact germinal vesicle. In the normal maturation process the mingling of the nuclear sap with the cytoplasm is very gradual, being completed in the case recorded not under ten minutes. It is this gradual mixing which apparently prevents disintegration.

Morgan ('93) and Mathews (Wilson and Mathews, '95) found that maturation was accelerated by shaking starfish eggs shortly after they were placed in sea water. They concluded that the shaking ruptured the membrane of the germinal vesicle and so allowed the nuclear material to mix more quickly with the cytoplasm. I have repeatedly tried to intermix cytoplasm and nuclear material by rupturing the nuclear membrane of the starfish egg with the needle, but in every case I get an explosive disintegration of the cytoplasm. The ruptured nuclear membrane which Mathews (W. and M., '95) and Marcus ('07) describe in fixed and stained immature eggs which had been violently shaken is possibly the membrane of the sphere which I found to persist after injury to the germinal vesicle (see page 321). It is more likely that the shaking which accelerates processes within the egg leads to the normal gradual dissolution of the nuclear membrane and the subsequent diffusion of the nuclear material throughout the egg. I have been able to do this occasionally with the needle. An intact germinal vesicle which to all appearances should take fifteen to twenty minutes to go into dissolution will often immediately exhibit a wrinkled outline on being gently agitated with the needle. Then follows the gradual fading from view of its outline with the subsequent changes as shown in Fig. 6.

The intact germinal vesicle may be brought into the sea water by tearing away the surrounding cytoplasm. During the process the nucleolus fades from view. The slightest tearing of the nuclear surface then causes the entire liquid vesicle to disappear in the water. If, however, the nucleus be left alone, it shrinks for a

time and then swells. The changes appreciable to the eye are shown in Fig. 7. During the swelling of the nucleus a substance apparently separates out which collects into a small mass and persists as a gelatinous body. It is possible that this abnormal separating out is analogous to the formation of the definitive egg nucleus in the normal process of maturation. This separating out of a gelatinous material from a liquid nucleus upon injury may be similar to the method of precociously inducing chromosomes in spermatocytes of the grasshopper (Chambers, '14).

2. The Existence of an Extraneous Membrane About the Unfertilized Egg.

The existence of a membrane about the unfertilized egg rising off as the fertilization membrane upon insemination was first suggested by the earlier investigators (*e.g.*, Hertwig, '76; Herbst, '93). Kite ('12) and Glaser ('13) agreed with them whereas McClendon ('14), Harvey ('14) and Elder ('13) claimed that the fertilization membrane is a new formation consequent to fertilization. Heilbrunn ('13) also identifies it with the actual protoplasmic surface of the egg, which he considers to be in a state of a gel and which lifts off as the fertilization membrane, a new surface film forming over the egg underneath it.

My experiments indicate that the unfertilized eggs of the starfish, sea-urchin and sand-dollar all possess a membrane extraneous to their true protoplasmic surface, and that it is this membrane which, upon insemination, is lifted off as the well-known fertilization membrane.

In the unfertilized egg the membrane is more or less tightly glued to the surface of the egg just as Kite ('12) described it. In the sea-urchin egg it is extremely delicate and can be demonstrated only as follows (Fig. 8): The needle is inserted as nearly as possible through the periphery of the egg and left there. Within a few seconds the protoplasm, lying immediately under the egg membrane and distal to the needle, flow away from the needle until the needle lies in a small protuberance which is formed by a very slightly lifted portion of the egg membrane.

The existence of the egg membrane is easily demonstrated in the

starfish egg. In Fig. 9 the disintegration of the cytoplasm following injury to the germinal vesicle has reached the surface of the egg. The disintegrated area is quickly localized by a surface film bounding a cup-shaped depression on the surface of the egg. Roofing over the depression is the egg membrane. The egg membrane can also be shown by cutting an egg in two by pressing the egg against the coverslip with the side of a needle. The pressure of the needle cuts the egg in two without rupturing the membrane, which, on releasing the egg, bridges the gap between the pieces and holds them together (cf. Figs. 11 and 12, page 329).

The difference between the consistency of the egg membrane in the starfish and the sea-urchin egg is strikingly shown in the fol-

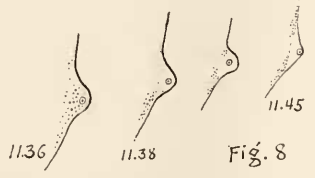

Fig. 8

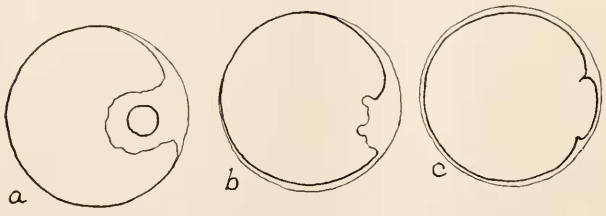

Fig. 9

FIG. 8. Needle inserted at 11:36 A.M. through periphery of a sea-urchin egg and left there. At 11:38 the cytoplasmic granules have been flowing away from the needle. A new surface film begins to appear with the needle left outside. At 11:45 the original egg membrane appears as a delicate membrane partially lifted off the surface of the egg by the needle.

FIG. 9. Lifting of a membrane from the surface of an immature starfish egg following injury to the egg. *a*, local disintegration of cytoplasm following destruction of the germinal vesicle (cf. Fig. 4). An egg membrane becomes apparent as the cytoplasm retreats from it. *b* and *c*, gradual separation of the membrane all over the surface of the egg.

lowing experiments. With the eggs in a hanging drop the egg is pressed against the coverslip with the side of a glass needle until

the pressure divides the egg into two pieces. In the sea-urchin egg the two pieces immediately round up and roll away from one another. In the starfish egg the tougher membrane is not ruptured, but holds the two pieces together.

The membrane of the sea-urchin egg is so delicate that it is also possible to cut the egg in two in the following manner: In a hanging drop the horizontal end of the needle is brought *over* the egg (Fig. 10). The needle is now lowered. This brings the needle

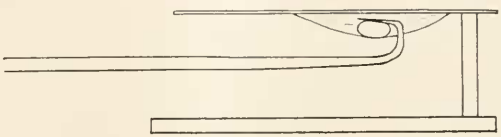

Fig. 10

Fig. 10. Side view of moist chamber to show one method of cutting an egg in two with the microdissection needle.

against the upper surface of the egg and presses the egg down against the surface film of the hanging drop. On lowering the needle still further it passes through the egg and out of the drop, cutting the egg cleanly in two. In the case of the starfish egg this procedure would drag the egg out of the drop along with the needle. The membrane of the sand-dollar egg is weaker than that of the starfish and stronger than that of the sea-urchin egg.

The consistency of the membrane varies with the age of the egg. The full-grown immature egg of the starfish has a relatively tough membrane. On the other hand, young ovarian eggs possess very delicate membranes and they can be cut in two with the same ease as mature sea-urchin eggs.

The strongest argument regarding the existence of a membrane about the unfertilized egg is that a membrane may be stripped off the egg whereupon the egg, which was previously non-adherent, now sticks to everything it touches. The fertilizability of such naked eggs is discussed under the next heading.

The existence of egg membranes is a fairly universal feature and it is, therefore, not surprising that we should find them in the

echinoderm eggs which have generally been considered as naked. The unfertilized *Cumingia* egg has an extremely tough membrane, so tough that it is difficult to rupture it without completely destroying the egg contents. The vitelline membranes in the frog and in the chick are undoubtedly analogous structures.

3. The Egg Membrane and the Fertilization Membrane are Identical.

Prior to fertilization no membrane enveloping the egg is visible. Upon fertilization a membrane lifts off which can easily be cut away from the egg. Figs. 11 and 12 indicate the identity of a preexisting membrane with the fertilization membrane. Fig. 11-*a* shows an egg cut in two with an investing membrane holding the pieces together. Upon fertilization the membrane lifts off, enclosing the two pieces in a single cavity (Fig. 11-*b*). One only of the pieces happened to segment, and the fact that the two pieces lie in one cavity is shown in Fig. 11-*c*, where the blastomeres of the segmented portion have encroached on the area around the nonsegmented piece. In Fig. 12 an egg was cut into three pieces, the egg nucleus lying in one of the pieces. Upon fertilization the membrane lifted off the pieces, each of which received sperm and developed into swimming larvæ. Fig. 12-*c* shows the empty fertilization membrane after the three larvæ had escaped. In Fig. 13 is shown an egg which, on being cut in two, was rolled about in an attempt to separate the pieces. The egg membrane between the two pieces was twisted into a thread joining the two. Upon fertilization each piece exhibited a complete fertilization membrane, but the fact that the two investing membranes are portions of one common membrane is shown by the connecting thread.

A conclusive test for the starfish and sand-dollar egg is the removal of the egg membrane prior to insemination. Occasionally, pricking the egg is sufficient to elevate the membrane. No subsequent development takes place. It is possible, however, to remove this membrane by tearing it and the egg then be made to slip out. This is more easily done on eggs which have been standing for some time in seawater. On catching at the sur-

face of such eggs with the needle, the membrane is often torn in such way that the egg slips out leaving the membrane stuck to the needle. Such an egg, when inseminated, is fertilized and subsequently segments with no investing membrane whatever.

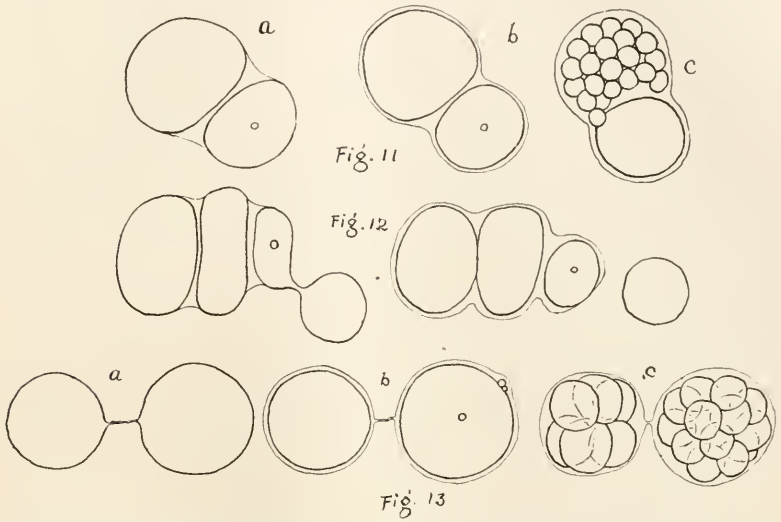

FIG. 11. *a*, starfish egg cut in two without destroying the investing membrane. *b*, after insemination the investing membrane lifts off both fragments as the fertilization membrane. *c*, one of the fragments segmented, the other did not. That both fragments lie in a common cavity is shown by the encroaching of blastomeres of one fragment into the region of the unsegmented fragment.

FIG. 12. *a*, starfish egg cut into three pieces. One piece was squashed and produced an exovate. *b*, on being fertilized the exovate was pinched off as an endoplasmic sphere (cf. Fig. 25). The rest of the fragments produced a common fertilization membrane. Each of the three enclosed fragments developed into a swimming larva.

FIG. 13. *a*, sand-dollar egg rolled as it was cut in two. The egg membrane between the two pieces was twisted into a thread joining the two. *b*, egg shortly after fertilization showing fertilization membrane about each connected by a filament. *c*, the two pieces in an early segmentation stage.

The difference in reaction of sperm to an egg which has been denuded of its membrane as well as of its jelly, and to one which has not is very striking. An egg within its membrane is quickly surrounded by spermatozoa as they are trapped in the jelly surrounding the membrane. In a membraneless egg no crowding of spermatozoa is noticeable and heavy insemination is necessary

to bring about fertilization. When a cloud of sperm has been blown upon a naked egg, one may frequently observe a spermatozoön swim toward it, wander over its surface, and then swim away. On the other hand, the empty membrane with its investing jelly immediately becomes covered with a halo of spermatozoa. This observation accords with the interpretation of Buller ('02), that the investing jelly determines the direction of the sperm which are captured by it, and that there is no apparent chemotactic substance excreted by the egg to attract the sperm.

The difference in position of the polar bodies in the starfish egg with respect to the fertilization membrane as shown by Gemmill ('12) (see also Chambers and Mossop, '18, and Garrey, '19) may be explained as follows: When the polar bodies form prior to fertilization they rise off the surface of the egg, carrying with them the closely adherent membrane. When they are pinched off the egg membrane remains continuous about the egg and subsequent insemination results in the formation of a fertilization membrane with the polar bodies lying outside. If, however, the eggs are inseminated before extrusion of the polar bodies, the egg membrane lifts off as the fertilization membrane and, when the polar bodies are formed, they lie within the membrane.

In the sea-urchin egg the identity of the egg membrane with the fertilization membrane is more difficult to demonstrate. In Fig. 14 is shown the effect of locally injuring the surface of the sea-urchin egg. In *a* is a disintegrated mass produced by tearing a spot on the surface with a needle. In *b* this area is shown as a bulge which may be explained as being produced by the interior pressure of the egg on a surface weakened by the loss of an investing membrane. In *c* the egg has been fertilized. The fertilization membrane is formed over all the surface except at the injured place. In *d* segmentation has occurred and a blastomere protrudes through the gap in the fertilization membrane.

A better demonstration is the case shown in Fig. 15. At 4:26 the tip of a needle was punched through the cortex. Within a few seconds the cytoplasm distal to the needle flowed away, leaving the needle lying under a delicate membrane (Fig. 15-*a*). At 4:27 the egg was inseminated with the needle still in place. At 4:29

the fertilization membrane was formed, showing its continuity with the delicate membrane previously noticeable (Fig. 15-*b*).

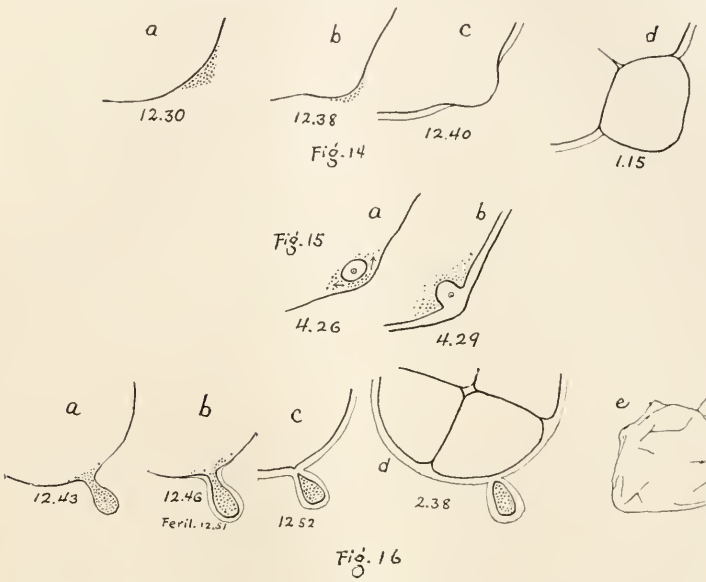

FIG. 14. Sea-urchin egg with surface torn producing local cytolysis. *a*, a new surface film has formed under the cytolyzed area which is being extruded. *b*, a bulge appears in the region of the new surface showing this region to be weaker than elsewhere on the egg surface. *c*, egg after fertilization exhibiting a fertilization membrane over the egg except at the place previously torn. *d*, the same egg 35 minutes later with a blastomere protruding through the tear.

FIG. 15. *a*, needle piercing sea-urchin egg near its periphery. The cytoplasmic granules are flowing in the direction of the arrows. One minute later the egg was inseminated. *c*, an intact fertilization membrane forms, inclosing both egg and needle tip.

FIG. 16. *a*, protrusion on surface of egg produced by pulling at cortex with needle. *b*, three minutes later the investing membrane lifted off surface of protrusion. *c*, one minute after fertilization. The protrusion has been pinched off from the egg and its investing membrane can be seen to be continuous with the fertilization membrane. *d*, empty and collapsed fertilization membrane.

In the sea-urchin egg the membrane often rises off a protrusion caused by pulling at the cortex with the needle. Such a case is shown in Fig. 16. The protrusion was formed at 12:43. At 12:46 a membrane had lifted off the protrusion. At 12:51 the egg was inseminated, and one minute later the membrane was

found continuous with the fertilization membrane. The protrusion subsequently pinched itself off and persisted in a sac-like protuberance of the fertilization membrane (Fig. 16-*d–e*).

In all of the various eggs studied a change in the consistency of the membrane takes place very soon after it has been elevated. The membrane, at first very soft and delicate, progressively toughens until it becomes almost parchment-like during the later segmentation stages. It is of interest to note that Harvey ('10) found a difference between the unfertilized and the fertilized sea-urchin egg when subjected to sulfuric acid. The acid dissolves the unfertilized egg completely, whereas it dissolves all of the fertilized egg except the fertilization membrane. Some chemical change apparently takes place as the membrane lifts off the egg.

Outside the membrane is a considerable zone of a structureless jelly. In the sand-dollar egg the jelly very loosely adheres to the membrane. On cutting into the jelly the egg with its membrane easily slips out. This is to a somewhat lesser degree true for the starfish egg. In the starfish egg one often sees the under surface of the jelly pushed away from the surface of the unfertilized egg by the protruding polar body.

The question as to whether the membrane lifts off the surface of the egg or whether the egg shrinks leaving the membrane behind has been raised by Glaser ('14) in spite of McClendon's ('10) statement to the contrary. Glaser, by making a large series of measurements, claims that the egg shrinks upon fertilization, and that the initial diameter of the completed fertilization membrane is equal to that of the unfertilized egg. Glaser's measurements were made on the assumption that the eggs always maintain a spherical shape. This is not true. The mature unfertilized egg is very soft and if allowed to lie on the bottom of a glass dish tends to flatten into the shape of a disc. Upon fertilization the egg rounds up as the fertilization membrane leaves its surface. One can readily see if the observations are taken of eggs in one plane only that erroneous conclusions may be arrived at.

I used two methods to ascertain the diameter of starfish eggs before and after fertilization. One method was to place a drop

containing a few eggs on a gelatin-coated slide. The eggs were rolled over by means of a micro-needle and only those which maintained their spherical shape were measured. With a micro-pipette sperm were introduced into the drop without disturbing the relative positions of the eggs. A second method was to place several eggs in a hanging drop in a Barber moist chamber. By piercing the surrounding jelly with a needle the egg to be measured could be held suspended in the middle of the drop. Numerous measurements of the starfish egg were made at different times through several summers and in every case the egg maintained its original size as the fertilization membrane rose off its surface. Not only does the egg not decrease in volume, but it slightly *increases* in size until segmentation occurs. The accompanying table is one sample of the measurements made:

	Unfertil.	Minutes after Fertilization.					
		1″	2″	7″	10″	20″	70″
Egg diameter.....	3.4	3.4	3.4	3.4	3.5 x 3.55	3.5 x 3.6	3.5 x 3.6
Fertilization membrane diameter.....		3.5	3.6	3.65 x 3.7	3.65 x 3.7	3.75 x 3.75	3.9 x 3.9

The conclusions from this table apply both to starfish and sea-urchin eggs. They may not necessarily be true for other species.

Fig. 17 shows successive steps in pulling a starfish egg out of its fertilization membrane. No second membrane is ever formed even with superimposed insemination. Occasionally the hyaline plasma layer in such an extruded egg swells up and simulates a second membrane, and it is probably this that has been described by certain investigators as a second fertilization membrane. The hyaline plasma layer will be discussed under heading 5.

An unfertilized mature sea-urchin egg may be rolled about and its contents churned to the extent of producing "fountain currents" within the egg (Chambers, '17-*b*). This is done by pushing an egg in a drop shallow enough to compress the egg. Currents are produced which flow backward immediately under the surface of the egg and forward along its central axis (Fig. 18). By careful manipulation it is possible to do this without rupturing

the investing membrane. Such an egg is capable of forming a normal fertilization membrane when inseminated. If the pushing process be carried too far, a distinctive quiver can be recognized, as of something giving way. On subsequent insemination such

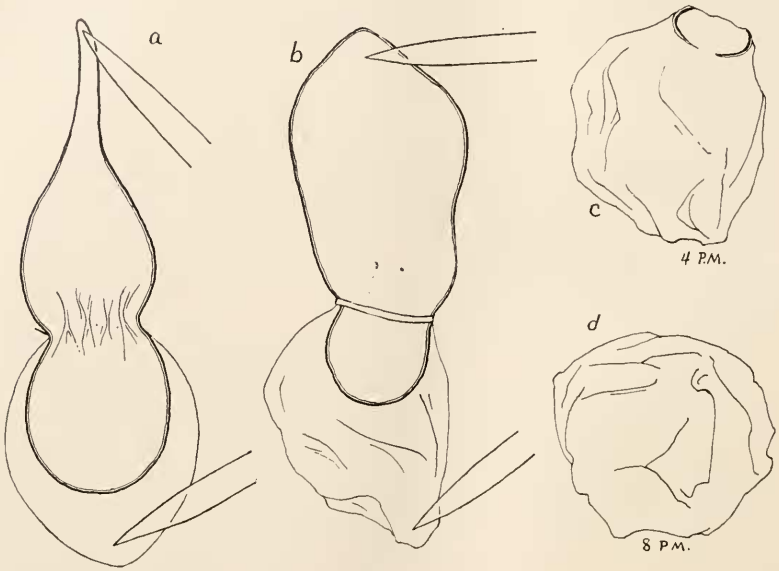

Fig. 17

Fig. 17. *a* and *b*, successive steps in pulling a starfish egg out of its fertilization membrane. *c*, empty membrane at 4:00 P.M. *d*, ditto four hours later at 8:00 P.M. The membrane persists as a collapsed remnant for a long time.

eggs produce a collapsed fertilization membrane. The quiver undoubtedly was due to a rupture of the egg membrane. On account of this rupture the fluid, which presumably collects under the membrane, leaks out and the membrane is not lifted uniformly.

4. The Cortex and Interior of the Unfertilized Egg.

The cytoplasm of the immature starfish egg is uniformly semi-solid. A gash made in it with a needle is maintained for some minutes before closing up. When the germinal vesicle breaks down naturally, the egg protoplasm becomes more fluid so that a gash

through such an egg quickly closes up. The cortex—*i.e.*, the surface of the egg immediately beneath the egg membrane—tends always to remain more solid (Chambers, '17-*a*). Because of this difference in consistency the cortex and medulla of the egg can be separated from one another as follows ('21[a]): If the surface of the mature starfish egg be torn with a needle and the egg then be caught at the opposite side and pulled to the edge of the

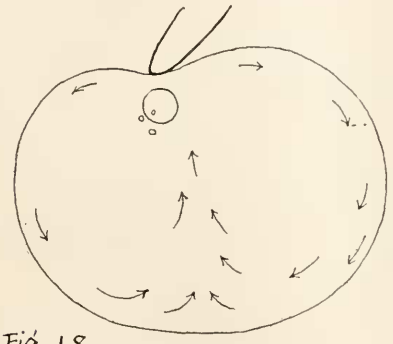

Fig. 18

Fig. 19

FIG. 18. Currents produced within a sea-urchin egg by pushing a sea-urchin egg held against a coverslip by a shallow film of water. The direction of the currents is shown by the arrows. The nucleus, after being carried about with the current, tends to come to rest in the location shown in the figure.

FIG. 19. Part of the cortex of a fertilized egg after the appearance of the hyaline plasma layer. The cortex was ruptured in one place and cytoplasmic granules can be seen issuing through the rupture in the hyaline plasma layer and the investing fertilization membrane.

hanging drop, the compression on the egg produced by the shallow water at the edge of the drop will cause the fluid interior to ooze out through the tear to form a spherical exovate (see Fig. 25, page 344). One may so manipulate the process as to cause the egg nucleus either to remain behind in the cortex (the cortical remnant) or to pass into the extruded sphere of endoplasmic material.

The cortical remnant is relatively solid and remains more or less inclosed within the egg membrane and its jelly. If left long enough it will eventually round up so as to present the appearance of a diminutive egg surrounded by a collapsed and wrinkled egg membrane.

The endoplasmic material which has escaped from the egg into the sea water is fluid and tends immediately to round up. On tearing with a needle its surface behaves like that of a highly viscous oil drop, adheres tenaciously to glass. As long as it possesses an intact surface it looks exactly like an egg fragment and will undergo disintegrative changes similar to those of entire eggs on being torn with the needle (cf. Chambers, '17-*a*).

The ability to produce endoplasmic spheres is possibly due to the relatively tough egg membrane in the starfish egg which helps to keep back the adherent cortex. In the sea-urchin egg, with an extremely delicate egg membrane, it has been impossible to cause the interior to flow out, as the cortex tends to flow with it.

The sand-dollar egg behaves very much like the starfish egg. The egg membrane is appreciable in the unfertilized egg and endoplasmic spheres are readily produced.

A difference in the functional activities of the cortex and interior of the starfish egg is discussed under the headings 6 and 7.

5. The Hyaline Plasma Layer.

Prior to fertilization the cytoplasmic granules in the sea-urchin and sand-dollar egg lie close to the surface. Within ten minutes after fertilization the granules have undergone a centripetal migration, leaving an appreciable peripheral zone of a hyaline appearance which has been called the hyaline plasma layer (Loeb's gelatinous film, '13, p. 19).

The microdissection needle indicates that this layer is relatively firm and gelatinous. The very fluid internal cytoplasm may be made to flow out through a rupture in this layer if the egg be torn. This is shown in Fig. 19. The cytoplasmic granules lie against the inner boundary of this layer and may be seen oozing out through the small tear in this layer and through a tear in the fertilization membrane to the exterior.

The hyaline plasma layer adheres very tenaciously to the needle and when an egg has been deprived of its fertilization membrane the egg sticks to everything it touches.

Loeb has called attention to the fact that the hyaline plasma

layer in a segmented egg bridges the segmentation furrow. When the furrow is first formed, however, the hyaline plasma layer does not bridge the furrow, but is carried in on the walls of the cleavage furrow (Fig. 20-*a*, *b*, *c*). The layer is thicker in the floor of the

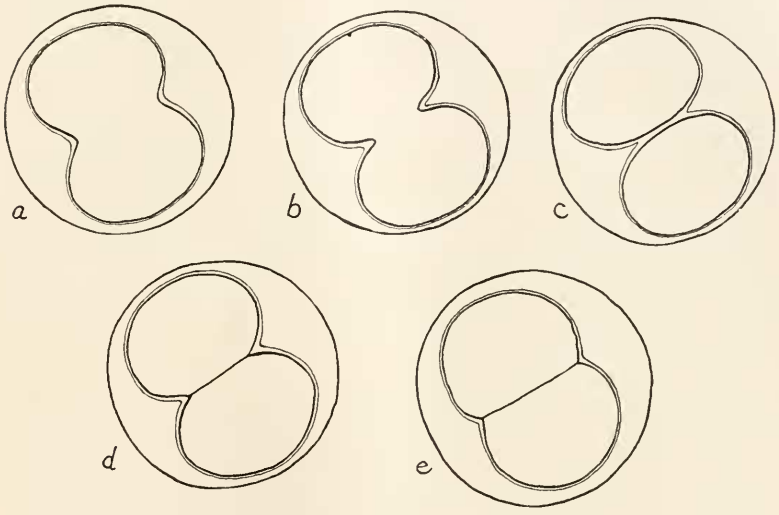

Fig. 20

FIG. 20. Contour of a sand-dollar egg at various stages of its cleavage into two blastomeres. In *a* and *b* the hyaline plasma layer is seen carried in on the walls of the deepening furrow. In *c* the egg has segmented in two with the hyaline plasma layer on opposite sides of the furrow tending to merge into each other. In *d* this process is carried further. In *e* the two blastomeres are tending to assume the shape of hemispheres with the hyaline plasma layer bridging the furrow.

furrow, but it is only later when the furrow has cut through the egg that the hyaline plasma layers on the opposite surfaces of the furrow run together. Each half of the segmenting egg tends to assume the shape of a sphere owing to the separation of the two asters of the amphiaster (Chambers, '17-*b*, '19). If there were no other forces at play, the two blastomeres, when formed, should be spheres. In the sea-urchin egg the adhesiveness of the hyaline plasma layer tends to draw the two blastomeres together; also the fertilization membrane, not rising to any great extent off the surface of the egg, must exert some pressure on the two blastomeres. In the sand-dollar the fertilization membrane is well

lifted, so that there is plenty of room within the membrane, permitting the two blastomeres to assume almost spherical shapes (Fig. 20-*c*). When the cleavage furrow is completed the two blastomeres are contiguous only where the two spheres touch. At this place the hyaline plasma layers of the two blastomeres merge. We have here, apparently, two opposing forces; first, the jellied aster holding each blastomere to a spherical shape, and, second, the affinity of the plasma layer substance surrounding the two blastomeres. As soon as the asters disappear and the cytoplasm of the blastomeres reverts to a more fluid state the plasma layers of the two blastomeres merge more and more and the blastomeres are pulled together till they assume shapes approaching those of hemispheres (Fig. 20-*e*). The outlines in Fig. 20 are camera lucida drawings taken during the successive stages of one sand-dollar egg.[1]

In the starfish, where there is no appreciable hyaline layer, and where the fertilization membrane is lifted far beyond the surface

[1] It has recently been intimated that the microdissection method is unreliable as a means of ascertaining changes in viscosity in the dividing egg because of supposed discrepancies in the results obtained by Seifriz ('20) and myself ('17[b] and '19). As a matter of fact the results of Seifriz harmonize perfectly with mine. Seifriz states "there is a pronounced decrease in viscosity of the central region of the cell with the first appearance of the amphiasters." This statement has been interpreted as running counter to mine. This is not true for although my results indicate that the astral portion of the amphiaster is jellied, I definitely state (p. 494, '17) that the central region and the zone between the two halves of the egg are fluid where "a distinct flow of granules medianward can be observed."

Again, on completion of cleavage Seifriz notes that the two blastomeres become liquid. This statement also fits in with my results. I state (p. 51, '19) that, immediately after cleavage and while the two blastomeres *are still spherical*, the firmness of the cytoplasm persists. Later, when the asters disappear the cytoplasm liquefies and the two blastomeres crowd up against one another. Seifriz noted this last liquid state of the two blastomeres without considering the state prior to it.

I may mention here a possible criticism of the centrifuge method in ascertaining viscosity variations. There are critical stages in the developing asters during which agitation causes their disappearance. This was noted long ago by Wilson. On bringing the eggs to rest the asters reappear and development proceeds normally. I have already discussed this matter fully ('19). The centrifuge and miscrodissection methods of studying the physical state of protoplasm should serve as valuable checks on one another, if only the investigators in these fields would agree on cooperation.

of the egg, the blastomeres are practically non-adhesive, and they maintain more or less spherical shapes till well on into the later segmentation stages.

6. The Localization of a Material Which Affects the Life of the Unfertilized Starfish Egg.

It is well known that immature starfish eggs can be kept in sea water at room temperature for 36 hours or more without disintegrating. That the germinal vesicle or nucleus is responsible for this length of life can be demonstrated by cutting an immature egg in two. The nucleated fragment lasts fully as long as the entire egg. The non-nucleated portion, on the other hand, disintegrates within three to four hours. In mature unfertilized eggs the conditions are quite different. In the mature egg the germinal vesicle has broken down and the nuclear sap has diffused throughout the egg. Loeb ('02) and Mathews ('07) showed that such eggs have a higher rate of oxidation than immature eggs and if left unfertilized disintegrate within 8 to 10 hours whereas the immature eggs last for days.

The non-nucleated fragment of the mature egg lasts as long as the whole egg, evidently owing to the dispersed nuclear sap of the dissolved germinal vesicle. What is significant is that the nucleated fragment lives no longer than the non-nucleated fragment. Both contain the dispersed nuclear sap, while the nucleated fragment possesses also the definitive mature egg nucleus which is ultimately to become the female pronucleus. Apparently it is the dispersed nuclear sap and not the definitive mature egg nucleus which is chiefly concerned. In the formation of the nucleus of the mature egg we have possibly something analogous to the state of affairs in many Protozoa where the nuclear apparatus consists of a tropho- or macro-nucleus concerned chiefly in the metabolic activities of the cell, and the kineto- or micro-nucleus which has only to do with the reproductive activities. In the starfish egg we may consider the germinal vesicle as a combined tropho- and kineto-nucleus. On the approach of maturation the tropho-nuclear material (nuclear sap) diffuses throughout the egg, leaving behind the kineto-nuclear part, the mature egg nucleus, which gives off the polar bodies to become ultimately the female pronucleus.

The fluid interior of the mature unfertilized egg, if isolated by being made to escape through a tear or the cortex, withstands disintegration for 24 to 36 hours. The presence of even a small part of the original cortex in organic continuity with it causes it to disintegrate in about the same time as an entire mature egg. This would indicate that the reactions which make for disintegration reside chiefly in the cortex. This, together with the fact that the cortex of the egg is necessary for fertilization, would indicate that the cortex is the seat of the initial activation processes of the egg. The relatively inactive central material of the starfish and sand-dollar egg somewhat resembles that of the *Linerges,* the Scyphomedusan, which Conklin ('08) has described. Conklin speaks of "the large cavity in the line of the first cleavage furrow filled with gelatinous or fluid substance, which forms the ground substance of the central area of the unsegmented egg." He found that most of the ground substance escapes into the cleavage cavity and suggested that it is the fluid yoke which is gradually used up in the nourishment of the embryo. The central substance of the *Linerges* egg is probably not strictly analogous with that of the starfish or sand-dollar egg. In *Linerges* cleavage is of a type peculiar to yolk-laden eggs and the central substance escapes during the first cleavage. On the other hand, in the echinoderm egg the nucleus lies well within the central substance of the egg and, upon fertilization, all of the endoplasm is used up in the formation of the cleavage asters and nothing apparently escapes into the early cleavage cavity. We can not, therefore, conclude that the interior of the Echinoderm egg consists of entirely inert material. It lacks certain essential features, but when coexistent with the cortex it plays a full part in the cleavage of the egg.

7. The Localization of a Substance Which Renders a Starfish Egg Fertilizable.

Wilson ('03[ab]) in *Cerebratulus* and *Renilla* and Yatsu ('04 and '08) in *Cerebratulus* have shown that non-nucleated fragments of the egg are capable of fertilization only after the germinal vesicle has broken down. With more delicate methods

rendered possible by the microdissection instrument it has been possible to work out this problem in detail and to ascertain to some extent the distribution of the material which renders fertilization possible.

A number of fully grown immature starfish eggs were enucleated by carefully dissecting out their germinal vesicles. None became fertilized when inseminated. In another lot of immature eggs the germinal vesicle was torn while in the egg (Fig. 21). Immediate

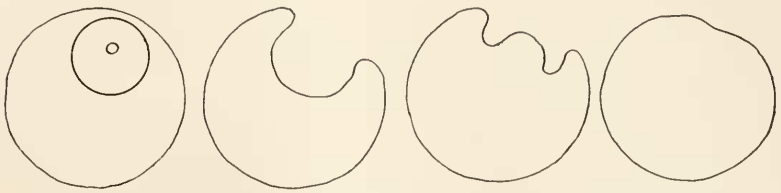

FIG. 21. A starfish egg whose germinal vesicle is eliminated by puncturing it (cf. Fig. 9). The cytoplasm surrounding this nucleus was also destroyed. This enucleated remnant is nonfertilizable.

dissolution of the nuclear membrane took place with a disintegration of the cytoplasm around the nuclear area. Those eggs which succeeded in forming a protective surface film to prevent spread of the disintegration process subsequently rounded up. Upon insemination none of the eggs showed any sign of being fertilized.

Eggs were then taken with the germinal vesicle in various stages of normal dissolution and cut into nucleated and non-nucleated portions. The eggs may be grouped into stages *b*, *c* and *d*, according to the stage of dissolution of their germinal vesicles, as shown in Fig. 6 (page 323). Whenever the cut passed through the nuclear area during the nuclear stages *b*, *c* and *d*, disintegration always took place, involving all of the nucleated portion and a small part of the non-nucleated piece (Fig. 23 *a*, *b* and *c*). When the cut did not pass through the nuclear area all persisting nucleated portions matured normally and upon insemination formed fertilization membranes and segmented. Of the non-nucleated portions those from eggs in stage *b* are non-fertilizable (Fig. 22). Those from eggs in stage *c* form fertilization membranes upon insemination. Nuclear division also takes place, so that the egg

fragment becomes multi-nucleated but remains unsegmented (Fig. 23-*c*). Non-nucleated fragments of eggs in a later stage (stage *d*) proceed somewhat farther (Fig. 24). The multi-nucleated masses arising from them make several periodic attempts at segmentation (Fig. 24-*c*). Small furrows appear over the surface of the egg, cutting in between the peripherally arranged nuclei.

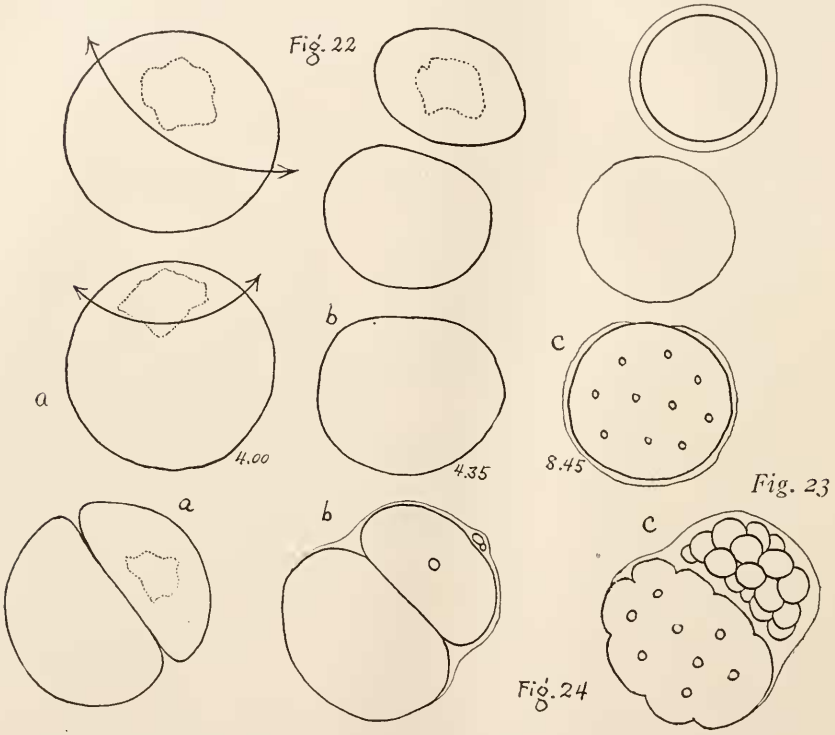

FIG. 22. Starfish egg in stage corresponding to *b* in Fig. 6 cut into two fragments. The non-nucleated fragment contains no material from the germinal vesicle and is nonfertilizable.

FIG. 23. Starfish egg in a later stage corresponding to *c* in Fig. 6 cut through the nuclear area. The cytoplasm in the injured nuclear area disintegrated leaving a non-nucleated fragment, *b*. That the fragment is fertilizable is shown in *c* by the formation of a fertilization membrane and the repeated division of the sperm nucleus. The fragment, however, is unable to segment.

FIG. 24. *a*, starfish egg in stage *d* of Fig. 7 cut into a nucleated and non-nucleated fragment. *b*, both fragments fertilized. The nucleated fragment segmented in the normal way with a number of blastomeres. The non-nucleated fragment became multinucleated and furrows appeared over its surface in an attempt at segmentation.

These furrows then disappear, to reappear again after a short interval. This may occur several times until the egg finally reverts to a spherical shape and remains so. In stage *f* the germinal vesicle has disappeared except for the definitive egg nucleus. Of such eggs any non-nucleated portion down to a certain size is capable of being fertilized and undergoing cleavage.

The above experiments lead one to infer the existence of a substance in the germinal vesicle which, on dissolution of the nuclear membrane, diffuses throughout the cytoplasm. The fertilizability of any egg fragment apparently depends upon the extent of diffusion of this substance. An egg fragment taken when a minimum amount of this substance has diffused into it will allow the sperm nucleus which has entered into it to divide. The presence of a little more of this substance will allow the fragment to undergo abortive segmentation. It is not until a sufficient amount is distributed throughout the egg that any fragment can develop properly.

Mature eggs were now studied, and it was found that any egg fragment in order to be capable of fertilization must contain a portion of the original cortex. The cortex and interior of mature unfertilized eggs were separated according to the method described under heading 4 (Fig. 25 *a* and *b*). The endoplasmic sphere and the cortical remnant were then inseminated. The fragment consisting of the cortical remnant is readily fertilizable and undergoes segmentation (Fig. 25 *b* and *c*). The endoplasmic sphere is non-fertilizable, no matter whether it contains the egg nucleus or not.

That the protoplasm of the endoplasmic spheres has not been irreparably injured in the process of flowing through a small tear in the cortex is shown in the following experiment. Eggs were squashed until the endoplasm protruded as lobate processes, whereupon the pressure on the eggs was lifted and the extrusion allowed to flow back into the egg. Such eggs are fertilizable and are capable of undergoing cleavage. One such case is illustrated in Fig. 26 where the cortex was torn in two places on squashing the egg and two exovates were formed. The nucleated exovate was allowed to pinch itself off. The other exovate flowed back into the remainder of the egg upon insemination (Fig. 26 *b* and *c*). A fairly com-

plete fertilization membrane formed around the egg except at the two torn spots and cleavage followed.

Endoplasmic exovates were also produced which remain connected by a bridge of protoplasm to the collapsed cortical portion

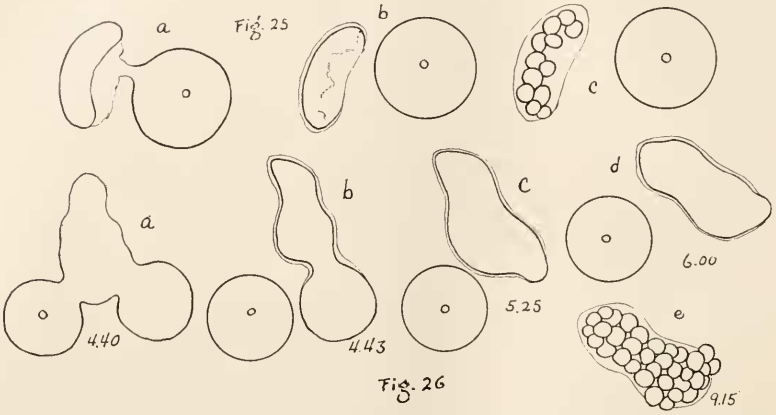

FIG. 25. *a*, nucleated exovate of internal cytoplasm produced by squashing a starfish egg. *b*, fragments inseminated after the endoplasmic sphere was pinched off. Only the ectoplasmic remnant forms a fertilization membrane. *c*, the endoplasmic sphere remains inert and nonfertilizable (cf. Fig. 12).

FIG. 26. *a*, starfish egg squashed producing two endoplasmic exovates. *b*, the nucleated exovate was pinched off. Upon insemination the other exovate drew back into the ectoplasmic remnant which formed a fertilization membrane. *c*, *d* and *e*, the ectoplasmic remnant underwent segmentation showing that the disturbance due to the squashing does not prevent segmentation. The endoplasmic sphere remains inert (*d*).

of the egg. On being inseminated the exovate either is drawn back into the cortical portion as the latter rounds up with the formation of a fertilization membrane or is pinched off, after which it remains as an inert body.

The possibility suggested itself that the substance which renders an egg fertilizable has a tendency to collect in the surface film of an egg and that, if an exovate remained in organic continuity with the egg, this substance might spread to the surface film of the exovate, thus rendering it fertilizable. Endoplasmic exovates were, therefore, produced which remained connected for varying lengths of time with the cortical portion of the egg. Some of the exovates remained connected for as long as fifteen minutes. Before insemi-

nation they were pinched off from the cortical portion of the eggs. None developed of those which were separated in such a way that there was no question as to their lacking any of the original cortex of the egg.

An endoplasmic sphere, in order to develop at all, apparently must incorporate in its substance at least a part of the original cortex of the egg. This is shown in Fig. 27. An exovate was

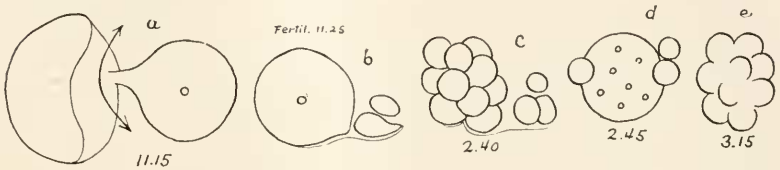

FIG. 27. *a*, an exovate is produced by squashing and most of the ectoplasmic part is cut away along line of arrow. *b*, the endoplasmic sphere formed itself incorporating a small part of the cortex. Upon fertilization the small cortical region formed a partial fertilization membrane. *c*, many furrows form simultaneously over the surface of the egg showing that it has been fertilized. (Note that the small cortical piece to one side of the egg has segmented in two.) *d*, the egg has reverted into a multinucleated nonsegmented mass except for three blastomere-like bodies which were pinched off. *e*, the fragment is again attempting to segment.

produced by crushing an egg (Fig. 27-*a*). However, before the exovate was set free most of the cortical remnant was cut away, leaving a very small piece which was drawn into the circumference of the endoplasmic sphere. On being inseminated a small shred of the egg membrane lifted off from this remnant, and this was all that constituted the fertilization membrane (Fig. 27-*b*). A sperm on entering this sphere underwent nuclear division several times. This was followed by cleavage furrows which formed on the surface of the egg between the peripheral nuclei and gave to the egg the appearance of a mulberry (Fig. 27-*c*). Some of the furrows deepened sufficiently to pinch off nucleated bodies. A few minutes later the furrows became obliterated and the main body of the egg appeared again as a non-segmented but multinucleated mass (Fig. 27-*d*). This process may occur several times (Fig. 27-*e*). The ability of an exovate to approximate normal segmentation is a function of the amount of the original egg cortex which it incorporates.

The inability of the endoplasmic sphere to develop is not due to the lack of successful sperm entry. Sections show that the sperm enter with ease but they remain unchanged and no asters form about them. In this regard the sperm react exactly as they do when they have entered immature eggs.

There must be something localized in the cortex which is necessary for successful fertilization and development (cf. Lillie, '14, '18). On the evidence presented here we may assume that this substance, originally within the germinal vesicle, diffuses out upon its dissolution and accumulates in the cortex of the egg. It is held in the cortex of the egg and is not carried out in the endoplasmic spheres on crushing the egg. The spheres are, therefore, incapable of being fertilized. Finally, the variation in the ability to segment among exovates containing varying amounts of cortical material indicates that there must also be a definite minimum amount of this substance present in order that an egg fragment may develop.

Conclusions.

1. The nucleus possesses a morphologically definite membrane.

2. Tearing the nucleus results in an immediate change of the nuclear membrane, followed by a disintegration of the cytoplasm surrounding it. This is most striking in the relatively large nucleus (germinal vesicle) of the starfish egg.

3. Injection of the germinal vesicle sap of one egg into the cytoplasm of another egg starts up disintegration processes in the injected area.

4. The mature egg nucleus can be pinched into two fragments. The fragments behave like fluid droplets and will run together when contiguous. Eggs whose nuclei have been operated upon in this manner are capable of normal segmentation.

5. A membrane can be demonstrated adhering to the surface of the unfertilized starfish, sea-urchin and sand-dollar eggs. This egg membrane is most pronounced in the starfish and least of all in the sea-urchin. In the starfish and sand-dollar the membrane can be stripped off without injuring the egg. In the starfish a very delicate egg membrane can be demonstrated investing half-sized

immature eggs. This membrane becomes more pronounced as the eggs reach their full growth and still more so as the egg matures. In the sea-urchin the immature eggs exhibit no trace of a membrane until the eggs begin maturation. In the mature unfertilized sea-urchin egg the membrane has reached a development comparable to that of the half-grown immature egg of the starfish.

6. The egg membrane rises off the surface of the egg upon fertilization and constitutes the fertilization membrane. No appreciable diminution in volume of the egg occurs during this process.

7. An egg, whose membrane has been removed, is fertilizable and segments without a fertilization membrane.

8. The hyaline plasma layer, which forms on the surface of the sea-urchin and sand-dollar egg within ten minutes after fertilization, binds the blastomeres together. In the starfish egg no such layer is formed, and, if the fertilization membrane be removed, the blastomeres tend to fall apart.

9. The fertilizability and approach to normal development of an egg fragment is directly proportional to the amount of a substance which emanates from the germinal vesicle during maturation.

10. The unfertilized mature egg possesses a more solid cortex of appreciable thickness inclosing a highly fluid interior. The fluid interior of the starfish and sand-dollar eggs can be made to ooze out through a tear in the cortex, whereupon it forms a surface film on coming into contact with sea water. In this way the internal and cortical material of the egg can be isolated from one another. Both round up, the internal material immediately and the cortical after some time.

11. Endoplasmic material, possessing a small part of the original cortex, is fertilizable and the approach to normal development is in direct proportion to the amount of cortical material present. The presence of even a small amount of cortical material causes disintegrative changes to set in at about the same time as in a whole egg.

12. The following table gives, for the various kinds of fragments of immature and mature starfish eggs, the length of time that they withstand disintegration when left standing in seawater and also whether they are or are not capable of being fertilized:

	Immature		Mature			
	Nucl. fragm. or entire egg	Non-nucl. fragm.	Nucl. fragm. or entire egg	Non-nucl. fragm.	Nucl. or Non-nucl.	
					Ectoplasmic remnant	Endoplasmic sphere
Longevity in hours...	24–36	2–3	8–10	8–10	8–10	24–36
Fertilizability...	+ (when mature)	−	+	+	+	−

As regards longevity it will be seen that the immature egg depends upon its nucleus (germinal vesicle) to prevent disintegration, for a fragment lacking the nucleus disintegrates very quickly. On the other hand, the mature egg, which has become permeated with the nuclear sap of the germinal vesicle, behaves quite differently. The non-nucleated fragment of a mature egg lasts longer than that of an immature egg and it is significant that the presence of the nucleus of the mature egg, which consists of not much more than the chromosomal constituents, has no effect in preventing disintegration.

The long period that the endoplasmic sphere withstands disintegration indicates that the factors which make for disintegration reside chiefly in the original cortex of the mature egg.

In regard to fertilizability it is evident that the substance which renders cytoplasm fertilizable emanates from the germinal vesicle and finally becomes localized in the cortex of the mature egg.

We can, therefore, distinguish three factors in the starfish egg; one affecting longevity, the second affecting disintegration and the third affecting fertilizability. The first and third have been traced to the germinal vesicle of the immature egg. The second is a function of the egg cortex.

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NOTE CONCERNING THE ORIGIN OF POLARITY IN THE FROG'S EGG. A CORRECTION.

A. W. Bellamy.

In connection with work published in 1919 (Biol. Bull., Vol. 37: 312–361) on the modification and control of development in the frog egg, it seemed desirable to determine if possible the origin of the polarity of the egg. The position taken was that polarity must be either a matter of inheritance or of determination by factors external to the egg. If the former possibility is true the problem is, of course, simply made more remote. The second possibility, since it is known or believed that polarity arises in a number of plant and animal eggs, in response to external factors, seemed the logical one to test, especially since it is the one most readily investigated experimentally. The first question was to determine the relation, if any, between the polarity of the egg and its mode of attachment to the ovarian membrane. Here it was found and it has since been confirmed, that in 75–80 *per cent.* of the cases, the pedicle which attaches the follicle to the ovarian membrane, is located on or within 20° of the equatorial region of the egg. Since a band 40° wide over the equatorial region of a sphere involves only about 34 *per cent.* of the total area it would seem that the pedicle is not located at random over the surface of the egg but with reference to some other factor, or factors.

Since the polar axes of the ovarian eggs bear every relation to gravity this factor is made highly improbable as having any influence on the origin of polarity.

The next question to arise was the relation of the polarity of the egg to its food and oxygen supply—the blood flowing through the follicular vessels. From observations made at that time on both injected and living specimens I believed and stated, p. 321, of the above mentioned paper, that " . . . in every case observed, the greater part of the arterial blood supply was restricted to the pigment hemisphere " and that the blood supply of the unpigmented hemisphere was largely venous. It was further sug-

gested that "the data indicate that polarity in the egg arises . . . in response to external conditions, viz., to the blood supply of the egg: that region of the oögonium chancing to be most richly supplied with arterial blood being destined to become, by virtue of this respiratory and nutritive relation, the animal pole of the egg."

It may be stated here that the problem was by no means considered solved and in 1919 plans for its further and more complete investigation were fairly well worked out. The investigation has continued with numerous interruptions and is still incomplete, but pending its outcome it has seemed desirable to make this statement.

It now appears that the previous observations were not sufficiently extensive to warrant the general statements indicated above. Certainly there is a considerable range of variation from what I thought was the typical situation and illustrated in Fig. 3 of the 1919 paper. And, it may be added, the figure is correct. But, on the other hand, cases have been observed more recently where the vegetative hemisphere was largely supplied by arterial blood, as well as various intermediate conditions. Furthermore one occasionally finds in the vessels that run to the follicle in the *mature* or nearly mature egg, a direct shunt between the small artery and vein. As far as the existence of any definite relation between the polarity of the *mature* or *nearly mature* egg and arterial or venous blood supply is concerned, I am obliged to withdraw the suggestion as it first appeared. It seems evident enough that polarity must be established early in the history of the egg, possibly in relation to the vascular supply. Supposing, as a working hypothesis, that such is the case it is conceivable that the vascularization in the follicle may change considerably especially as the egg approaches maturity—the only stage previously examined. It is along these lines that the investigation is being continued with the hope of throwing further light on the question.